

KHAN, MOHAMMAD MAZAMAL, M.S. Inhibition of Cytochrome P450 2E1 and Cytochrome P450 2A6 by Essential Oils: Tarragon (*Artemisia dracunculus*) and Basil (*Ocimum basilicum*). (2014)
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Cytochrome P450 enzymes are involved in the metabolism of foreign substances that are present within living organisms. These P450s are classified as Phase I metabolizing enzymes that are found mainly in the liver, they belong to a superfamily of heme containing monooxygenases that are required for metabolism for a number of xenobiotics. Mechanistic approach by these enzymes involves carrying out the oxidation of carbon and nitrogen groups usually resulting in the addition of an alcohol. Cytochrome P450 enzymes have been involved in the bioactivation of inactive compounds to active electrophiles that can contribute to the production of reactive oxygen species, such as superoxide, free radicals, and peroxides¹. These ROS can contribute to oxidative stress, which leads to hepatic cell necrosis, lipid peroxidation, and DNA adduct formation.

This study focuses on two specific Cytochrome P450s, CYP2E1 and CYP2A6. These enzymes have shown to have one of the highest rates of uncoupling among all P450s, this uncoupling leads to the production of ROS. This study focuses on finding potential inhibitors that decrease the chances of these uncoupling reactions from occurring. Human, rat, and rabbit liver microsomes were used for these studies. A series of essential oils were screened with these P450s to determine their inhibitory affects on the enzyme. From these studies, tarragon (*Artemisia dracunculus*) and basil (*Ocimum basilicum*) showed the highest inhibitory affects on the enzyme. The major constituent for both these oils was estragole, this was also confirmed via GCMS testing, because of

this it was thought that it was a major reason to why the enzyme activity is being inhibited. With the initial screening data showing inhibition of CYP2E1 and CYP2A6, next goal was to determine how potent these oils were and what is the mode of inhibition, reversible or irreversible. The results of CYP2E1 and CYP2A6 with human, rat, and rabbit concluded that both essential oils and estragole inhibited the activity of the enzyme. Estragole was the most potent inhibitor of both enzymes in all 3 species. The K_i value for estragole with CYP2E1 in humans was 22.4 μM , in rat 143 μM , and in rabbit 108 μM . The K_i value for estragole with CYP2A6 in humans was 27.5 μM and in rabbit was 49.3 μM . Results show that the mode of inhibition shown by both these essential oils and estragole was non-competitive and a reversible type of interaction occurred with both Cytochrome P450 enzymes.

INHIBITION OF CYTOCHROME P450 2E1 AND CYTOCHROME P450 2A6 BY
ESSENTIAL OILS: TARRAGON (*ARTEMISIA DRACUNCULUS*) AND BASIL
(*OCIMUM BASILICUM*)

by

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Approved by

Committee Chair

To my Mother, Father, Family, and Friends
Without your encouragement this would not have been possible,
Thank you for your love and support.

APPROVAL PAGE

This thesis written by Mohammad Mazamal Khan has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

1.1.0 Cytochrome P450 Enzymes and Metabolism

At any given time there are a number of foreign substances that are present within living organisms and the metabolism of these xenobiotics is important to maintain a homeostatic equilibrium. Enzymes are essential in the metabolism of these xenobiotics; there are two classes of enzymes that are involved in this process. They are described in general terms as Phase I and Phase II enzymes. Phase I is involved in oxidation and phase II in conjugation. Reactions of both phases cause compounds to become more hydrophilic, thus making it simpler for them to be readily eliminated through renal excretion. An important class of Phase I enzymes are the Cytochrome P40 enzymes, which belong to a superfamily of heme containing monooxygenases that are required for metabolism of number of endogenous and exogenous compounds. Xenobiotic metabolizing Cytochrome P450s are found in the liver and a variety of other mammalian tissue¹. The overall mechanistic view is that Cytochrome P450s carry out the oxidation of carbon and nitrogen groups usually resulting in the addition of an alcohol.

Cytochrome P450s are responsible for metabolism of most xenobiotics that enter the body. However, these enzymes are also involved in the bioactivation of inactive

compounds to active electrophiles that can be potential toxins to the liver¹. When certain chemicals are bioactivated they can lead to cell necrosis and sometimes to cellular transformation that eventually results in cancer or in some instances damage to hepatic tissue. One such example of this is when acetaminophen is bioactivated to a highly reactive Quinone, N-acetyl-p-benzoquinone (NAPQI). NAPQI can produce reactive oxygen species (ROS) and covalently bind to cellular nucleophiles such as DNA, RNA, and proteins, which eventually results in cellular necrosis. Acetaminophen is known to cause liver failure (through hepatic necrosis) when taken in high concentrations, which leads to hepatotoxicity².

1.2.0 Properties of Cytochrome P450s

There are approximately 60 Cytochrome P450 genes in the human body³. All Cytochrome P450 gene names start with a CYP, representing that they belong to the P450 family. Following this is a number, which specifies which gene family the enzyme is coming from, next is a letter representing the gene's subfamily, and at the end a number is assigned to distinguish the specific gene within the subfamily. An example of this would be if we had a CYP P450 gene that is in-group 2, subfamily E, and gene 1 would be written as CYP2E1.

With multiple studies showing how Cytochrome P450s are involved in the activation of xenobiotics, it is beneficial to reduce activity of such enzymes so the creation of toxic intermediates is decreased⁴. In order to fully understand the mechanistic properties of these enzymes it is important to study their molecular structure. The active

site is important in determining activity of these isoforms. A commonality between all CYP P450s enzyme is that they contain an iron atom at the core of the heme within the porphyrin ring. The reduction of this iron atom is the rate-limiting event of the overall reaction⁵.

1.2.1 Cytochrome P450 Mechanisms

The catalytic cycle of P450s is essential to understanding how these isoforms function. Figure 1 is a depiction of the general reaction of all Cytochrome P450 enzymes. Starting from the first step of the mechanism, the substrate comes into the active site of the enzyme, which causes the displacement of the water molecule that is bound to the iron center. This displacement of water causes the ferric Fe^{III} complex to be reduced, by an electron that is donated from Nicotinamide adenine dinucleotide phosphate (NADPH), to Fe^{II} . Once this reduction occurs, it allows molecular oxygen to come in and bind to the iron center, forming an iron-oxo complex. Upon the formation of this complex, it allows NADPH to donate a second electron to the system that reduces the oxygen. The oxygen molecule then goes through protonation that allows one of the oxygen molecules to be cleaved and leave the system in the form of a water molecule. Upon this an oxo-ferryl intermediate is formed (represented in step 6), this intermediate is highly reactive. The highly reactive oxo-ferryl complex allows the oxidation of the substrate to occur. Once the oxidation of the substrate happens, this allows a water molecule to come in and displace the newly formed product, and the catalytic cycle starts over again. In figure 2 it can be seen how this same catalytic cycle can lead down a different pathway when

uncoupling is exhibited. This leads to the production of reactive oxygen species such as peroxides and superoxide. ROS's lead to oxidative stress, which ultimately can cause damage to cells, lipid peroxidation, and DNA adduct formation. Looking at figure 2 it can be seen how the dissociation of the peroxo-iron complex from stages 4 and 5 lead to the formation of this ROS's. Once the peroxide and superoxide dissociates the iron goes is free to accept more electrons.

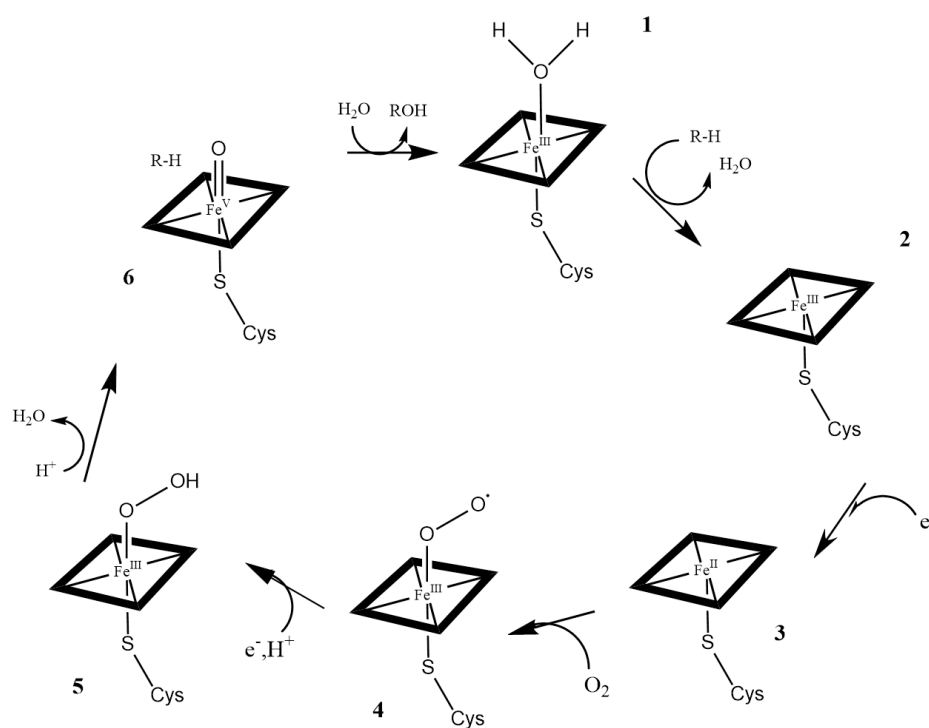


Figure 1. Catalytic Cycle of Cytochrome P450s. The reaction depicts what happens from the point of entrance of the substrate into the active site. The overall outcome of this reaction is that the substrate is hydroxylated so it can be readily excreted from the living organism.

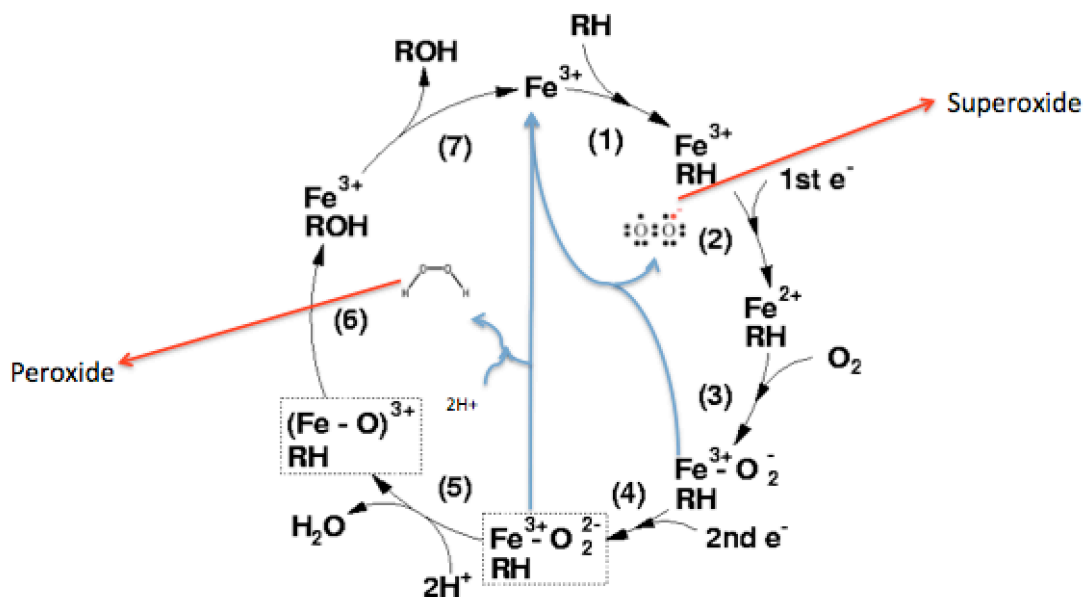


Figure 2. Uncoupling with CYP P450s. The catalytic cycle showing how reactive oxygen species are produced through uncoupling of iron-oxo complex in stages 3 and 4 of the mechanism.

1.2.2 O-Demethylation Reaction

CYP P450s carry out various types of chemical oxidation reactions that are dependent upon the substrate that is present. The o-demethylation reaction is one common reaction carried out by these P450 isozymes. This reaction involves a removal of a methyl group that is attached to an oxygen atom. As shown in figure 3, the substrate is hydroxylated via the P450 catalytic cycle. The position of the hydroxyl group causes an unstable hemi-acetal to form, which then rearranges and causes the demethylation reaction to occur.

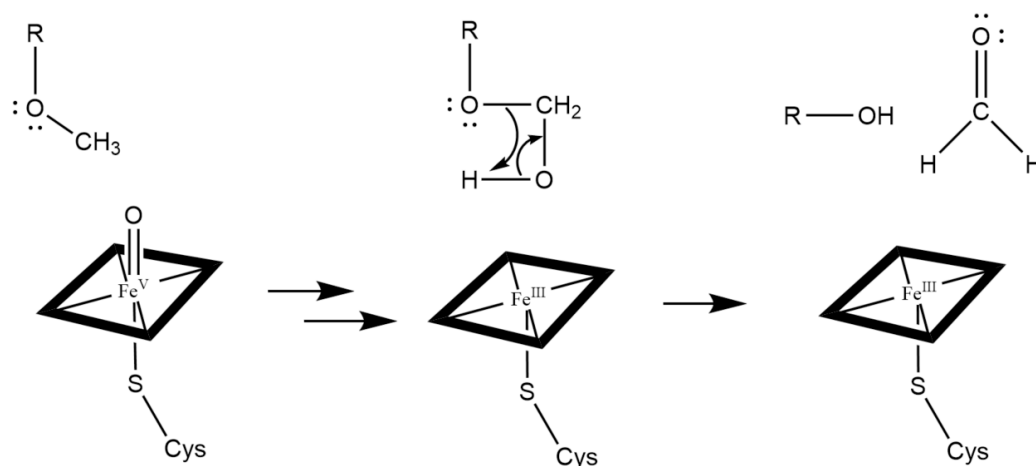


Figure 3. O-Demethylation Reaction. It can be seen oxo-ferryl intermediate generated from the CYP P450 catalytic cycle is involved in oxidizing the terminal methyl group of the substrate. This forms the unstable hemi-acetal, which then rearranges causing the demethylation, forming formaldehyde.

1.2.3 Terminal Olefin Reaction

Substrates involving terminal olefins can go through a slightly different mechanistic pathway. Terminal olefins are involved in a nonconcerted olefin epoxidation pathway⁶. Through this reaction, terminal olefins are not only oxidized to the corresponding epoxides but also involved in alkylating one of the prosthetic heme nitrogen atoms, as seen in figure 4. Mechanistically, since this is a nonconcerted reaction, the epoxide does not alkylate the heme group. The heme alkylation occurs before the formation of the epoxide metabolite, resulting deactivation/inhibition of the P450.

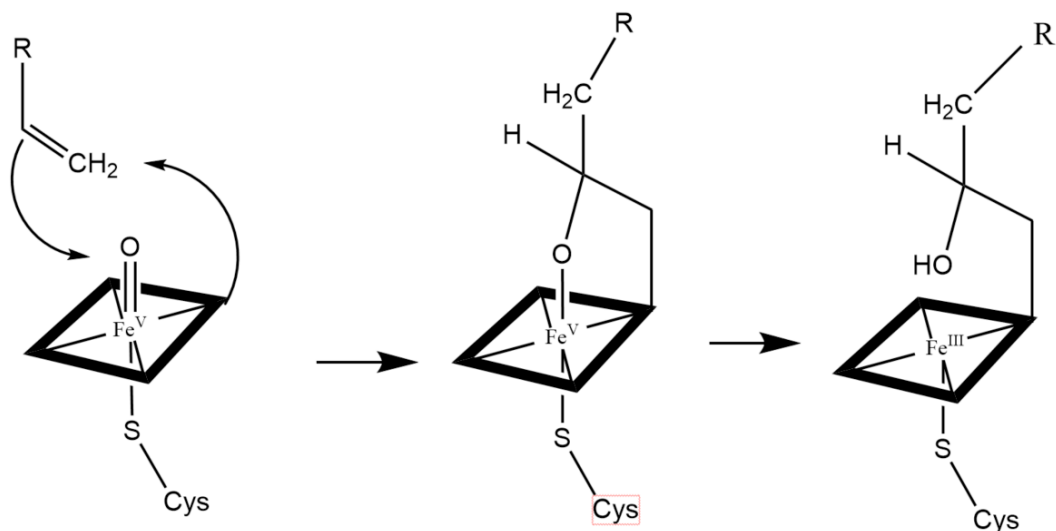


Figure 4. Terminal Olefin Reaction. The reaction involves the alkylation of nitrogen from the heme group of the P450 enzyme during the oxidation of the terminal olefin.

1.2.4 Benzene Hydroxylation Reaction

Aromatic compounds are common substrates that are catalyzed by CYP P450s.

As shown in figure 5, the mechanism involves the removal of hydrogen from a benzene ring. Upon being oxidized via the P450 catalytic pathway, an epoxide forms. An NIH shift is observed, which allows the aromaticity to be restored and the benzene to be hydroxylated to give a final product of phenol.

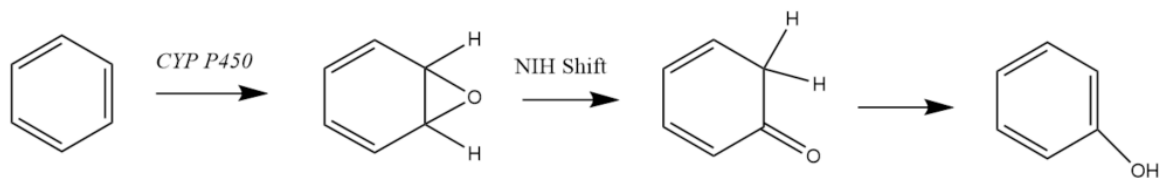


Figure 5. Benzene Hydroxylation Reaction. The arene oxide is formed from the metabolism of benzene via the catalytic cycle; from this an NIH shift is observed which eventually leads to a hydroxylation of the benzene to a phenol⁷.

1.3.0 Inhibition of Cytochrome P450s

It is vital to understand the interactions of natural products and CYP P450 isozymes. Natural products are being used to treat illnesses in an increasing number by people in the United States⁸. For this it is important to understand how these natural products interact with CYP P450 enzymes. Natural products consist of many small molecules that can influence enzymatic activity drastically. To understand these affects it is important to determine the mode of inhibition from these chemical compounds involving CYP P450s.

The Michaelis-Menten model is widely used to determine enzyme activities with CYP P450s. As seen in figure 6, the Michaelis-Menten model illustrates the relationship between enzyme and substrate concentration. The V_{\max} represents the maximum rate of product formation after the saturation of all available enzymes by the substrate. The other variable that can be determined from this model is K_m , which represents $\frac{1}{2} V_{\max}$. The K_m indicates where 50% of the substrate is bound to the enzyme. With the introduction of

inhibitor this model can be used to describe the nature of the interaction between P450 and molecules that inhibit the enzyme.

Enzymatic activity can be altered in the presence of inhibitors. An inhibitor consists of any molecule that restricts the enzyme from its function. As stated before Cytochrome P450s maybe involved in generating ROS's, with the presence of inhibitors with certain xenobiotic interactions this process can be limited or in some case completely neutralized. The focus of this study is to focus on compounds found in natural products and their inhibitory affects on CYP P450s. There are two types of inhibitors, irreversible and reversible, and they are classified by their mode of binding to the enzyme.

Reversible inhibitors compete with the substrate and bind to the active site, thus preventing the substrate from binding to the active site. The effects of a reversible inhibitor on the enzyme only last as long as it's present in the system⁹. Once the inhibitor is removed from the environment normal activity will resume. The strength of these inhibitors is determined by there binding affinities. Inhibitors bind to the active site via interactions with amino acids within the active site or by hydrogen binding¹⁰.

Irreversible inhibitors bind to the enzyme covalently. These types of inhibitors chemically alter the enzyme shape in such a way that it permanently inactivates the enzyme. Such inhibitors are sometimes referred to as "suicide inhibitors". Mechanistically irreversible inhibitors mode of action on CYP P450s usually involves chemical modification of the heme cofactor, such as the terminal olefin example described above¹¹.

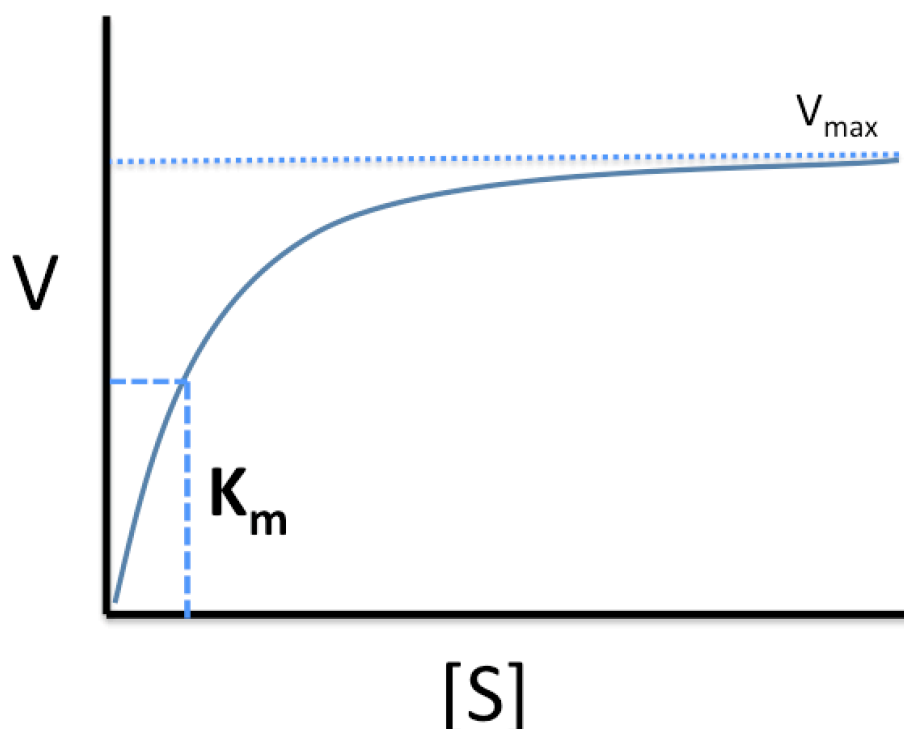


Figure 6. Michaelis-Menten Schematic. The Michaelis-Menten model, showing the relationship between reaction rate and substrate concentration. The V_{\max} represents the maximum rate at which product is formed, and K_m represents $\frac{1}{2} V_{\max}$.

1.4.0 Role of CYP 2E1 in Oxidative Stress

Oxidative stress results from the increase of intracellular prooxidant species such as hydrogen peroxide, hydroxyl radicals, and superoxide anion radical¹. Elevated levels of these molecules can go on to cause mitochondrial damage, DNA modifications, lipid peroxidation, elevated cytokine production, and eventually cell death. In mammals, ROS can have serious implications such as Alzheimer's disease, atherosclerosis, cancer, diabetes, chronic obstructive pulmonary disease, and neurological disorders¹².

Oxidative stress is believed to be one of the fundamental reasons why the process of aging occurs¹³. CYP2E1, as mentioned before is involved in generating high levels of ROS in vitro and in vivo. During the catalytic cycle, CYP450s use a hydride from NADPH to reduce oxygen, which leads to the production of hydrogen peroxide and superoxide anion radical. When the process is uncoupled from the catalytic cycle, this causes the release of these ROS. Studies have clearly shown that in the absence of substrate, the 2E1 isoform is proficient in generating ROS through this route^{14,15}. This occurrence is called futile cycling. It occurs because P450-derived NADPH oxidase activity is independent of exogenous xenobiotic substrates¹⁷. CYP2E1 expression is said to be elevated with the induction of non-alcoholic steatohepatitis (NASH), which results in oxidative stress⁹. ROS that are produced as a result of CYP2E1 can produce lipid peroxides from polyunsaturated fatty acids, and these react with other cellular macromolecules causing cell toxicity and death¹.

1.4.1 Cytochrome P450 2E1

A goal of this study is to identify natural products that selectively inhibit CYP2E1. In particular the study focuses on commonly used essential oils. CYP2E1 is mainly located in the liver but is also found in areas such as the lungs, brain, and other tissue¹⁸. Studies have shown that CYP2E1 levels are elevated in the liver when alcohol levels are high. This could possibly be an explanation to why alcoholics develop liver disease¹⁹. The enzyme is involved in inducing oxidative stress that can potentially be extremely detrimental to human health. CYP2E1 is found to metabolize *p*-nitrophenol

into nitrocatechol with a high binding affinity. The active site of CYP2E1 is relatively hydrophobic and small compared to other CYP P450 enzymes. It is reported that CYP2E1 is one of the smallest active sites that exists in the CYP450 family. The overall size of the active is 190 angstroms, which explains why the substrates and inhibitors for CYP2E1 are small in molecular weights²⁰. CYP2E1 has nonpolar phenylalanine residues that make upper part of the active site, which explains why it interacts with aromatic compounds such as *p*-nitrophenol. In figure 7, the reaction of CYP2E1 oxidizing *p*-nitrophenol to nitrocatechol is represented. The identification of inhibitors to inhibit CYP2E1 is important because of its ability to induce oxidative stress. In alcohols such as ethanol, CYP2E1 plays a vital role in metabolizing it to a more reactive and toxic product acetaldehyde. Studies have shown that if CYP2E1 levels decrease then alcohol induced liver damage also decreases⁶. Compounds such as diallyl sulfide (DAS), phenethyl isothiocyanate (PIC), and chlormethizole have been shown to inhibit CYP2E1, and have been shown to reduce the damaging effects of 2E1-dependent metabolism in-vivo²¹. From this it can be concluded that natural products can be effective inhibitors of CYP2E1, which may prevent oxidative stress from occurring under certain conditions.

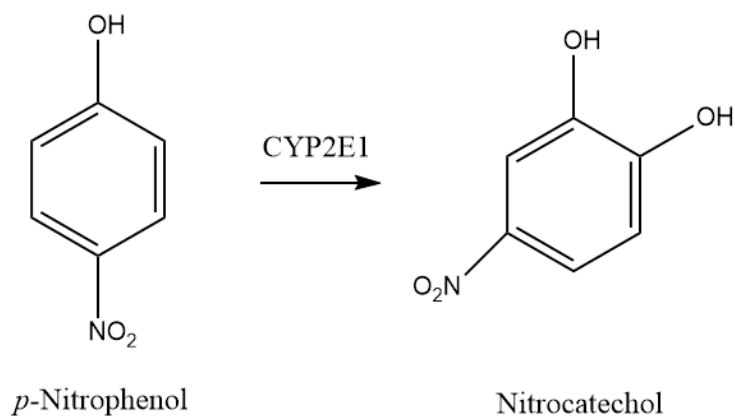


Figure 7. Reaction of CYP2E1 with *p*-Nitrophenol. The reaction causes the *p*-nitrophenol to be oxidized to nitrocatechol.

1.5.0 Cytochrome P450 2A6

Cytochrome P450 2A6 (CYP2A6) is the most abundant of the cytochrome P450 2A family located in the human body²². It is expressed in moderate levels in hepatic cells, localized within the endoplasmic reticulum. CYP2A6 is not a major drug-metabolizing enzyme, studies show that it metabolizes less than 5% of all drugs⁶. One of the major roles of CYP2A6 is detoxification of nicotine in the human body. CYP2A6 studies tend to focus on its involvement in the metabolism of xenobiotics and potential production of carcinogenic intermediates. CYP2A6 metabolizes coumarin to 7-hydroxycoumarin, as shown in figure 8. Since it is the only enzyme that carries out this reaction, it is ideal to use it as a substrate for CYP2A6. The active site of CYP2A6, like CYP2E1, is small and hydrophobic²³. For this compactness, it only allows small planer and hydrophobic substrates to enter, making coumarin an ideal choice. When coumarin is in the active site,

the carbonyl oxygen hydrogen bonds with the Asn297²³. Asn297 interacts with the coumarin and holds it in such away that it can be oxidized. Another significant interaction arises with the presence of Phe107 in the active site; this amino acid residue interacts with the aromatic aspect of the substrate. These interactions show why coumarin is an ideal substrate for CYP2A6.

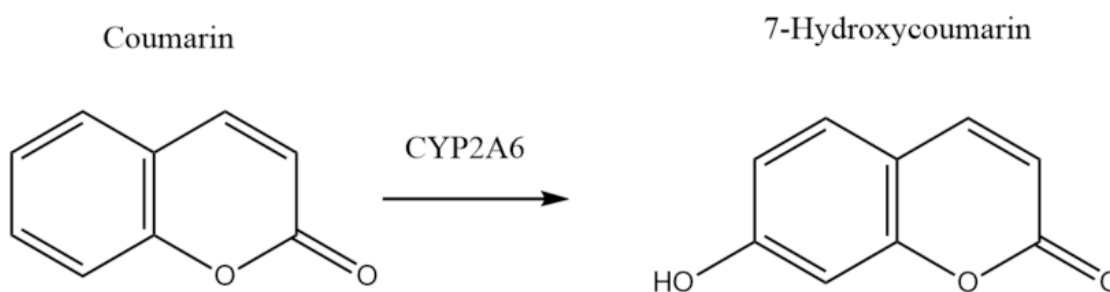


Figure 8. Reaction of CYP2A6 with Coumarin. The reaction causes coumarin to be catalyzed over to 7-hydroxycoumarin.

1.5.1 CYP2A6 Role in Metabolism

Harmful substances that are located within cigarettes are also bioactivated by CYP2A6, which are potentially cancer-causing agents²⁴. Thus inhibition of this isoform may also result in a protective effect under specific environmental conditions. CYP2A6 is involved in the metabolism of nicotine that enters the body, to cotinine²⁵. Inhibited CYP2A6 could potentially be used as an approach for smoking cessation²³. As mentioned before, CYP2A6 is known to bioactivate chemical compounds into cancer causing ones, nitrosamines being a key example of this activation. It was found that patients that had an absence of the CYP2A6 enzyme had a lower risk of lung cancer when compared to the

ones that had the enzyme present²⁶. Therefore inhibition of this enzyme may also reduce the risks of lung cancer.

1.6.0 Essential Oils

Essential oils have proven their usefulness throughout human history for multiple purposes, such as aromatherapy, medicinal, and for culinary uses. Essential oils that were used in this study are *Artemisia dracunculus* (Tarragon) and *Ocimum basilicum* (Basil). While these oils contain a vast array of constituents, they both have a significant presence of estragole (1-allyl-4-methoxybenzene).

1.7.0 Objective Statement

The purpose of this study is to determine the activity of CYP2E1 and CYP2A6; an in vitro assay was developed to measure the activity of these enzymes using hepatic microsomes from rabbits, rats, and humans. The substrate, *p*-nitrophenol was selected for CYP2E1 and coumarin for CYP2A6. These substrates were chosen because of their specificity to binding to the enzyme. The strategy employed was to screen a series of essential oils for inhibition of these enzymes.

Tarragon and basil were selected because of their inhibitory effects on enzymatic activity. Studies will include identifying which component in the oil is/are responsible for the inhibition, and what type of inhibition is occurring (irreversible or reversible), and from this a mechanism will be proposed of how this potential inactivation of the enzyme is transpiring.

CHAPTER II

EXPERIMENTAL

2.1.0 Preparation of Reagents

2.1.1 Phosphate Buffer and NADPH

Potassium phosphate buffer was prepared by mixing monobasic potassium phosphate and dibasic potassium phosphate, both of which were purchased from Carolina Biological Supply Company. A 1 M stock solution at pH 7.4 was used for all assays involved in this project. NADPH was purchased from Research Products International. It was then diluted with Nano-pure water and partitioned into 10 mM stock solution aliquots. These aliquots were stored in a freezer at -80°C.

2.1.2 Preparation of Rabbit Liver Microsomes

Rabbit liver microsomes were used in this study to obtain a comparative analysis (human and rat being other hepatic microsomes) across species. Rabbit liver microsomes were generated in lab. The rabbit liver was first washed in 0.05 M potassium phosphate buffer at pH 7.4, which also comprised of 0.1mM of EDTA. Rabbit liver and buffer solution was then blended to obtain a homogenized mixture. Next the liver was centrifuged at 5,000 rpm for 10 minutes. The pellets were then discarded and the supernatant was centrifuged at 37,000 x g for 1 hour. The microsomes were then

homogenized in a solution of potassium phosphate buffer at pH 7.4. Lastly, the microsomes were put in 200 μ L aliquots and stored in the -80°C freezer for usage.

2.1.3 Preparation of Rat Liver Microsomes

Rat liver microsomes were also prepared in lab (as part of the comparative analysis across species). The rat liver was first rinsed in a solution that comprised of 100mM potassium phosphate buffer at pH 7.4, 0.1 mM EDTA, 20% glycerol, 3.3 mM $MgCl_2$. Then the liver and buffer were put into a blender to mix and obtain a homogenized mixture. The blended mixture was then centrifuged at 5,000 rpm for 10 minutes. The supernatant was then again centrifuged but this time at 25,000 x g for 1 hour. Upon the completion of this, the supernatant was removed and the pellets were homogenized in potassium phosphate buffer at pH 7.4. Lastly, the microsomes were put in 50 μ L aliquots and stored in the -80°C freezer for usage.

2.1.4 Human Liver Microsomes

Human liver microsomes were purchased from Molecular Toxicology Inc. They were put in 50 μ L aliquots and stored in -80°C freezer for usage.

2.1.5 Preparation of *p*-Nitrophenol

The reagent *p*-nitrophenol (*p*-NP) was purchased from Sigma-Aldrich. 13.9 mg of *p*-NP was weighed out and dissolved in 10 mL of deionized water to give 10 mM stock solution. This solution was then diluted 1:10, to give a final concentration of 1 mM stock

solution of *p*-NP, which was used as a substrate for all assays that involved CYP2E1 enzyme.

2.1.6 Preparation of Coumarin

The reagent Coumarin was purchased from Sigma-Aldrich. 15 mg of the Coumarin was weighed and dissolved in 10 mL methanol solution; this is done because of Coumarins low solubility in water, this gives a 0.01 M stock solution. Then this solution was diluted 100X to give a final concentration of 100 μ M, which was used in all assays involving CYP2A6.

2.1.7 Preparation of Nash Reagent

The Nash reagent was used to identify the formation of possible formaldehyde from the metabolism of estragole by CYP2E1 and CYP2A6. The reagent was prepared by mixing 15g of sodium acetate, 200 μ L of 2,4-pentandione, and 400 μ L glacial acetic acid to give a total volume of 50 mL. The solution was vortexed for 5 minutes and stored in a low light environment in the 4°C refrigerator.

2.2.0 CYP P450 Assay Development

The study involves measuring product formation by measuring different parameters such as microsomal and substrate concentration, time, and inhibitor affects on CYP2E1 and CYP2A6. Inhibition studies were carried out to determine the inhibitory effects of tarragon and basil on the enzyme. These two oils have a common substituent in

high concentrations named estragole, which was also separately tested to study the kinetic effects on the enzymes.

2.3.0 Initial Screening Assay for CYP2E1

CYP2E1 was tested with various essential oils to determine their inhibitory effects on the enzyme. Primarily the goal was to identify essential oils with strong inhibitory potency towards the enzyme. For CYP2E1, *p*-nitrophenol was picked as a substrate for the enzyme, which produces nitrocatechol (as seen in figure 7). Essential oils were diluted 5 μ L in a 100 mL of deionized water to make the stock solution. In a 1.5mL centrifuge tube a reaction mixture containing 0.1M potassium phosphate buffer (pH 7.4), liver microsomes (from rat \sim 15.3 μ g/mL, rabbit, and human-23.3 μ g/mL), 1mM *p*-nitrophenol, and various concentrations of the oils were prepared. Deionized water was added to bring the final volume up to 200 μ L per sample. The reaction was vortexed and then initiated with the addition of 1mM NADPH and incubated for 30 minutes at 37⁰C. After 30 minutes, the reaction was quenched using 6% perchloric acid, vortexed, and then put into an ice bath for approximately 6-8 min. The reaction was taken out of the ice bath, and centrifuged for 8 minutes at 14,000 g to remove the precipitate. Then the supernatant was transferred into an HPLC vial for HPLC analysis.

2.3.1 HPLC Analysis

The reactions involving *p*-nitrophenol oxidation were examined using HPLC (high-performance liquid chromatography). The analysis was conducted using a Shimadzu Automated liquid Chromatography system with UV detection at 340nm. The system was programmed to inject 75 μ L of each sample onto a C18 column (4.6x150mm) with a flow rate of 1.5 mL/min. The mobile phase used for this method contained 40% acetonitrile, 60% deionized water with 0.1% trifluoroacetic acid. The nitrocatechol peak from each reaction was integrated to give peak areas, where the areas under the curves represent the relative activity of the CYP2E1 enzyme found in rat, rabbit, and human.

2.3.2 CYP2E1-Determination of K_i

Enzyme kinetic studies were carried out using hepatic microsomes from human, rat, and rabbit with tarragon, basil, and estragole. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.4, varying concentrations of substrate, *p*-NP, ranging from 10 μ M-100 μ M, hepatic microsomes, a specified concentration of inhibitor which ranged from 0-23.00 μ g/mL, and nanopure water was added to bring the reaction volume up to 200 μ L. After all components of the reaction were added, the vials were vortexed, the 0.5 mM NADPH was added to the reaction mixture and placed inside an incubator at 37⁰C for 30 minutes. After 30 minutes, the reactions were quenched with 6% perchloric acid and placed in an ice bath for 8 minutes. Samples were then centrifuged for 8 minutes at 14,000 g, to remove the precipitate. 200 μ L of the supernatant was then placed inside of an HPLC vial insert for HPLC analysis.

An important tool for studying enzyme kinetics is the Michaelis-Menten model. This model is used to determine the apparent K_i for each inhibitor. K_i was found by plotting V vs. $[S]$ at a range of increasing concentration of the substrate, p -NP, in both the presence and absence of the inhibitor. The inhibitor concentration was chosen from screening experiments carried out on the oils where ~50% inhibition of CYP2E1 was observed. The Slide Write Plus (by Advanced Graphics Software, Inc.) program was used to plot the relative activity vs. concentration of p -NP. The Michaelis-Menten equation, as seen in equation 1, was used to determine the maximum velocity without inhibitor present, V_{\max} , with inhibitor present, V_{\max}^{app} . The substrate concentration at which the enzymatic activity is $\frac{1}{2}$ the V_{\max} , represented by K_m , and the substrate concentration with the presence of inhibitor when the enzyme is $\frac{1}{2}$ the V_{\max}^{app} , which is represented by K_m^{app} .

$$V_o = \frac{V_{\max} * [S]}{K_m + [S]}$$

Equation 1. The Michaelis-Menten Equation.

Using Equation 2, the K_i was calculated using the concentration of inhibitor.

$$V_{\max}/V_{\max}^{\text{app}} = 1 + [I]/K_i$$

Equation 2. Equation for Calculating K_i for Non-Competitive Inhibition.

2.3.3 Two Stage-Reversibility Studies Assay

With preliminary studies showing definite inhibition of CYP2E1 using tarragon and basil, the next phase was to look at how the enzyme was inhibited. To determine if the inhibition was irreversible or reversible, a method using two stages was applied. The experiment was done with multiple parameters (altering the components in stage I). Multiple parameters were constructed by changing the volume of microsomes, changing the concentration of inhibitor, and changing pre-incubation time (stage I) and incubation time (stage II).

Table 1-A, 1-B, show the parameters used for each stage I experiment. 20 μ L from the stage I vials was transferred to stage II of the experiment. In stage II, the vials had 20 μ L from each respective vial from stage I, 20 μ L of potassium phosphate buffer, 10 μ L of *p*-nitrophenol, 20 μ L of NADPH, and 130 μ L of deionized water. Final volume of the reaction in stage II was 200 μ L. In the vial where D was transferred, inhibitor (1-3 μ L depending upon reaction conditions) was added into the stage II reaction vial.

Vial A in this experiment represented reaction conditions which would allow the inhibitor to damage the P450, if the reaction is irreversible then there would be no/or less remaining activity toward the substrate when the 20 μ L is transferred to stage II, but if its reversible then there would be full activity with the substrate in stage II to the P450. NADPH is also added in vial A to see if the inhibition is dependent upon NADPH. In vial B, everything is the same as A, except there is no addition of NADPH. Vial C represents a control, with just microsomes and buffer, when transferred over to stage II part of the reaction, the substrate should bind to the P450 to give full activity (which then can be

compared with Vial A and B). Vial D, is the same as Vial C, but in stage II part of the reaction, the inhibitor is added before 20 μL of Vial D is transferred. This is to ensure that any inhibitor carried over into stage II does not inhibit the reaction.

From Table 1-A, two reaction conditions were setup. The first reaction involved taking the vials prepared in stage I and leaving them out at room temperature for 30 minutes then transferring 20 μL to stage II reaction, where it was then incubated at 37°C for 30 minutes. The reaction was then quenched with 200 μL of 6% perchloric acid. The second reaction condition involved taking the vials prepared in stage I and leaving them out at room temperature for 10 minutes and then transferring 20 μL to stage II reaction, where it was then incubated at 37°C for 30 minutes. The reaction was then quenched with 200 μL of 6% perchloric acid. The results for both of these experiments can be seen in figure 18A-B.

From Table 1-B, two reaction conditions were setup. The first reaction (Figure 19A-B) involved taking the vials prepared in stage I and leaving them out at room temperature for 12 minutes then transferring 20 μL to stage II reaction, where it was then incubated at 37°C for 30 minutes. The reaction was then quenched with 200 μL of 6% perchloric acid. The second reaction condition involved taking the vials prepared in stage I and leaving them out at room temperature for 36 minutes, then transferring 20 μL to stage II reaction, where it was then incubated at 37°C for 45 minutes. The reaction was then quenched with 200 μL of 6% perchloric acid. The reason the reaction duration was increased was because it was believed that the kinetic rate of the reaction might be slow

thus increasing the time might enhance any irreversible type inhibition. The overall reaction setup can be seen in Figure 9.

Table 1-A. Two-Step Reversibility Study-First Parameter. This was used to determine if the reaction is irreversible or reversible. The inhibitor used was estragole: initially 5 μL of oil was dissolved in a 100mL of deionized water to make a stock solution. The concentration of inhibitor used was 9.33 $\mu\text{g/mL}$.

Vial A:	Vial B:	Vial C:	Vial D:
15 μL of human liver microsomes	15 μL of human liver microsomes	15 μL of human liver microsomes	15 μL of human liver microsomes
5 μL of potassium phosphate buffer	5 μL of potassium phosphate buffer	5 μL of Buffer	5 μL of Buffer
5 μL of NADPH	5 μL of Deionized Water	15 μL of Deionized Water	15 μL of Deionized Water
10 μL of Inhibitor	10 μL of Inhibitor	-	-

Table 1-B. Two-Step Reversibility Study-Second Parameter. This was used to determine if the reaction is irreversible or reversible. The volume of human liver microsomes was tripled from 15 μL to 45 μL in the initial incubation. The inhibitor (estragole) concentration was tripled, 15 μL of inhibitor was diluted in 100mL of deionized water. Thus the concentration of inhibitor used was 21.2 $\mu\text{g/mL}$.

Vial A:	Vial B:	Vial C:	Vial D:
45 μL of human liver microsomes	45 μL of human liver microsomes	45 μL of human liver microsomes	45 μL of human liver microsomes
15 μL of potassium phosphate buffer	15 μL of potassium phosphate buffer	15 μL of Buffer	15 μL of Buffer
15 μL of NADPH	15 μL of Deionized Water	25 μL of deionized water	25 μL of deionized water
10 μL of Inhibitor	10 μL of Inhibitor	-	-

Reversibility Study-Assay Development

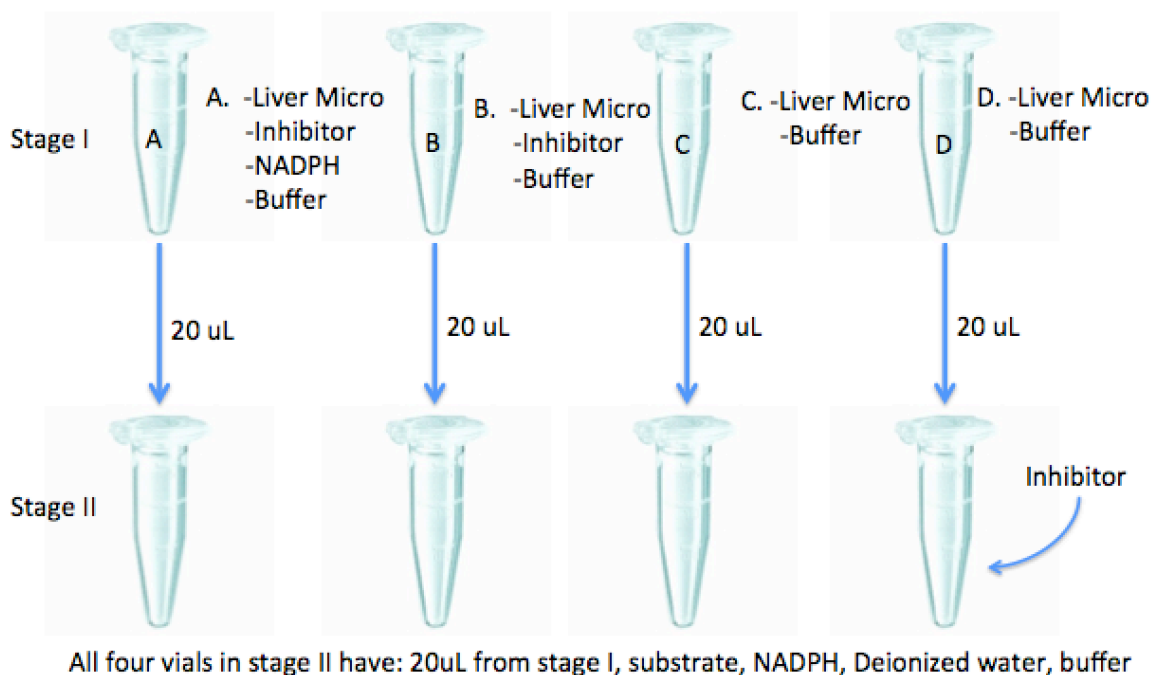


Figure 9. Two-Stage Reversibility Study Setup.

2.3.4 Time Dependent Studies Assay-CYP2E1

Time dependent studies were done to determine the affects of the essential oils on CYP2E1 over time. This was also another method used to determine if the reaction was reversible or irreversible. The reaction conditions for this assay involved hepatic microsomes (human, rat, and rabbit), 0.1 M Potassium Phosphate buffer, pH 7.4, 50 μ M of substrate, *p*-NP, 5.8 μ g/mL of tarragon and basil, for estragole 40 μ M were used (these concentration was used because this is where the enzyme is ~50% inhibited), also a control was run without inhibitors, and 115 μ L of nanopure water was added to a vial. 0.5

mM NADPH was added to initiate the reaction, also bringing the reaction volume to 200 μ L. The reaction was quenched with 6% perchloric acid at 10, 20, 40, 60, and 80 minutes and then placed in an ice bath for 8 minutes. Samples were then centrifuged for 8 minutes at 14,000 g, to remove the precipitate. 200 μ L of the supernatant was then placed in an HPLC vial insert for HPLC analysis.

2.4.0 Initial Screening Assay for CYP2A6

CYP2A6 was tested with various essential oils to determine their inhibitory effects on the enzyme. Primarily the goal was to identify essential oils with strong inhibitory potency towards the enzyme. For CYP2A6, coumarin was picked as a substrate for the enzyme, which produces 7-hydroxycoumarin (as seen in figure 8). Essential oils were diluted 5 μ L in a 100 mL of deionized water to make the stock solution. In a 1.5mL centrifuge tube a reaction mixture containing 0.1M potassium phosphate buffer (pH 7.4), 20 μ L liver microsomes (from rat, rabbit, and human), 50 μ M 1mM p-nitrophenol, and various amounts of each oil was prepared. Deionized water was added to bring the final volume up to 200 μ L per sample. The reaction was vortexed and then initiated with the addition of 0.5 mM NADPH and incubated for 30 minutes at 37⁰C. After 30 minutes, the reaction was quenched using 6% perchloric acid, vortexed, and then put into an ice bath for 8 min. The reaction was taken out of the ice bath, and centrifuged for 8 minutes at 14,000 g to remove the precipitate. Then the supernatant was transferred into an HPLC vial for HPLC analysis

2.4.1 HPLC Analysis

The reactions involving coumarin oxidation were examined using HPLC (high-performance liquid chromatography). The analysis was conducted using a Shimadzu Automated liquid Chromatography system with UV detection at 320nm. The system was programmed to inject 75 μ L of each sample onto a C18 column (4.6x150mm) with a flow rate of 1.0 mL/min. The mobile phase used for this method contained 40% acetonitrile, 60% deionized water with 0.1% trifluoroacetic acid in both reagents. The 7-hydroxycoumarin peaks from each reaction was integrated to give peak areas, where the areas under the curves represent the relative activity of the CYP2A6 enzyme found in rabbit and human livers.

2.4.2 CYP2A6-Determination of K_i

Hepatic microsomes from human and rabbit with tarragon, basil, and there major constituent estragole were used to carry out enzyme kinetic studies. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.4, varying concentration of substrate, coumarin, ranging from 1 μ M-10 μ M, hepatic microsomes, a specified concentration of inhibitor that ranged from 0-23.00 μ g/mL, and nanopure water was added to bring the reaction volume up to 200 μ L. After all components of the reaction were added, the vials were vortexed, 0.5 mM NADPH was added to the reaction mixture and placed inside an incubator at 37⁰C for 30 minutes. After 30 minutes, the reactions were quenched with 6% perchloric acid and placed in an ice bath for 8 minutes. Samples were then centrifuged for

8 minutes at 14,000 g, to remove the precipitate. 200 μ L of the supernatant was then placed inside of an HPLC vial insert for HPLC analysis.

The Michaelis-Menten model was used to study the activity of the enzyme. Equation 1 was used to determine the V_{\max} , V_{\max}^{app} , K_m , and K_m^{app} . Michaelis-Menten model was used to determine the type of inhibition and the potency of the inhibitor on the enzyme. The K_i was calculated for a non-competitive inhibitor using Equation 2. K_i was calculated for both the presence and absence of inhibitor in the reaction.

2.4.3 Time Dependent Studies Assay-CYP2A6

To determine the mode of inhibition, reversible or irreversible, time dependent studies were done with tarragon, basil, and estragole on CYP2A6. The reaction conditions for this assay involved hepatic microsomes (human, rat, and rabbit), 0.1 M Potassium Phosphate buffer, pH 7.4, 5.0 μ M coumarin, 5.8 μ g/mL of tarragon and basil, for estragole 40 μ M were used (these concentration were used because this is where the enzyme is ~50% inhibited), also a control was run without inhibitors, and 115 μ L of nanopure water was added to a vial. 0.5 mM NADPH was added to the initiate the reaction, also bringing the reaction volume to 200 μ L. The reaction was quenched with 6% perchloric acid at 10, 20, 40, 60, and 80 minutes and then placed in an ice bath for 8 minutes. Samples were then centrifuged for 8 minutes at 14,000 g, to remove the precipitate. 200 μ L of the supernatant was then placed in an HPLC vial insert for HPLC analysis.

2.5.0 Nash Assay

The Nash Assay is a method used to measure the formation of formaldehyde. In this study the Nash assay was used with CYP2E1 and CYP2A6 to determine if they were metabolizing estragole to form the formaldehyde product via demethylation. The Nash reagent includes ammonium acetate and 2,4-pentanedione, and acetic acid. The reagent reacts with the formaldehyde to give 3,5-diacetyl-2,6-dihydrolutidine, which gives a yellow color. The yellow color can be quantified to determine how much formaldehyde forms. The appearance of the yellow color in this study would indicate that the P450 has carried out the O-Demethylation reaction (figure 3).

CHAPTER III

RESULTS AND DISCUSSION

3.1.0 CYP2E1 Enzyme Activity

Several parameters were tested in order to optimize the bioassay for testing activity involving CYP2E1. This process involved testing varying concentration of enzyme, substrate concentration, and incubation time. Rat, rabbit, and human liver microsomes were tested for their enzymatic activity. The assays were prepared using similar conditions as mentioned previously with equivalent concentrations of potassium phosphate buffer, substrate (PNP), and NADPH, but with no inhibitor. Figure 10 shows how varying concentration of microsomes were tested with the substrate, *p*-nitrophenol, to show its effects on the formation of nitrocatechol. As expected, increase in microsomal concentration resulted in a corresponding linear increase of product formation in the 30-minute reaction. From this it was determined that 20 μ L of the rat liver microsomes, 20 μ L of rabbit Liver, and 5 μ L of the human liver microsomes produced sufficient activity for use in this study.

CYP2E1 microsomes were then tested to determine the linearity over time. Activity should increase in a linear fashion with the increase of reaction time, assuming no inactivation occurred. As shown in figure 11, the effects of reaction time on CYP2E1 activity again followed a predictable linear dependence out to nearly 1-hour incubation time. From this it was determined that an incubation time of 30 minutes was appropriate.

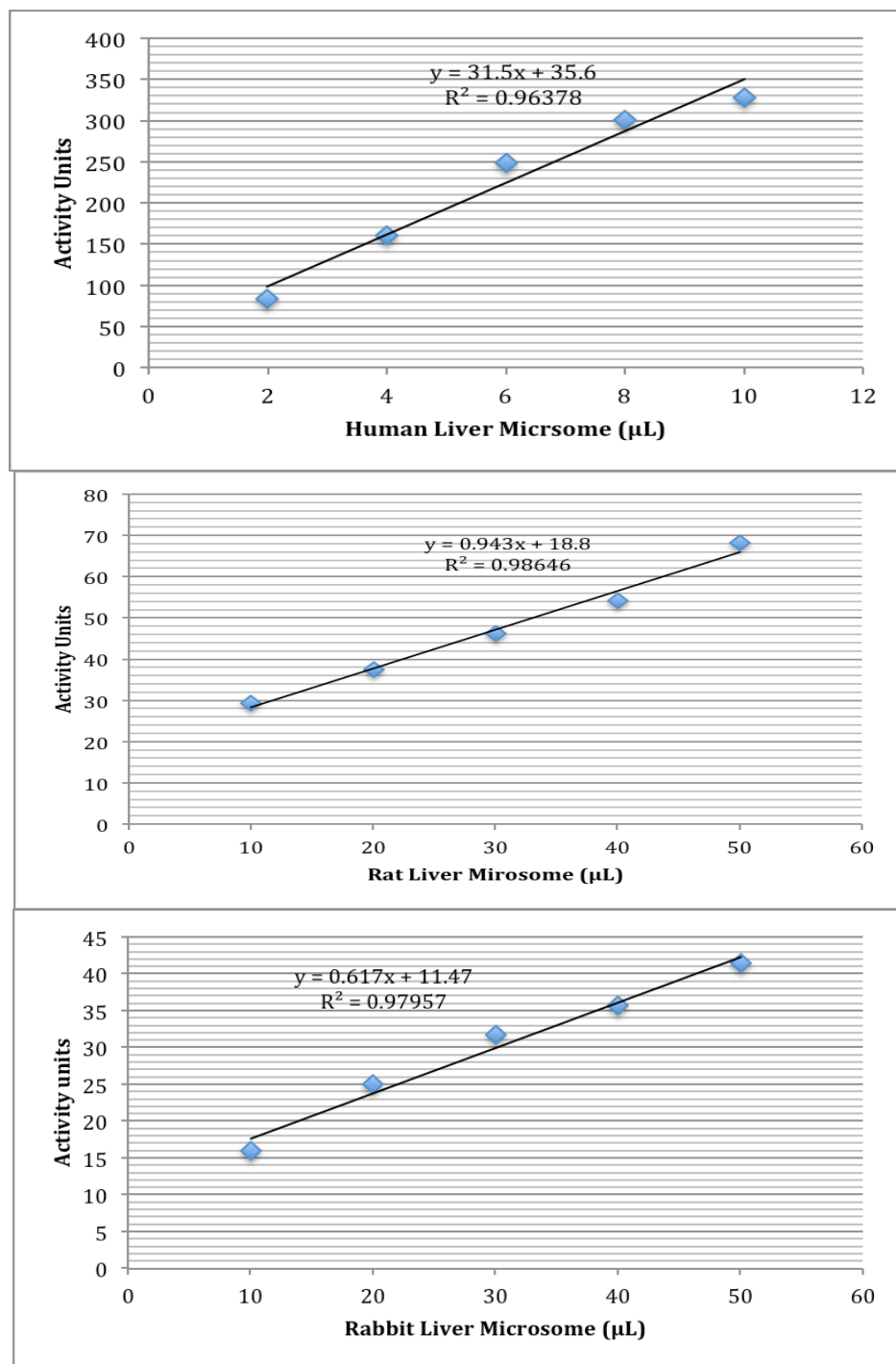


Figure 10. Results for Variation in Microsomal Concentration-CYP2E1. From this it was determined the amount of microsomes used for each species for their respective bioassay.

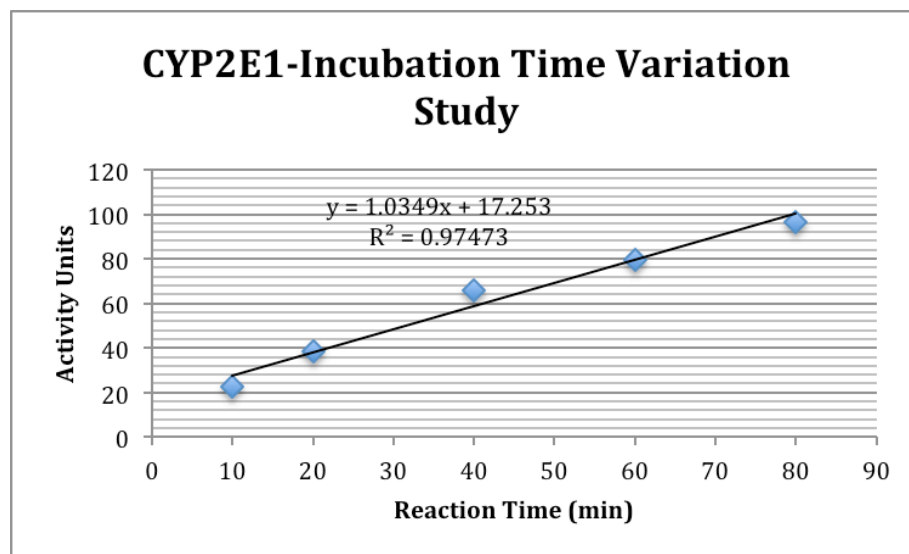


Figure 11. Results for Variation in Incubation Time-CYP2E1. From this it was determined that the appropriate time for the bioassays will be 30 minutes.

CYP2E1 was tested using different concentrations of substrates to determine the Michaelis-Menten enzyme kinetic parameters. Through this analysis it was determined that the K_m value for the substrate to be approximately 50 μM (data not shown).

3.1.1 Inhibition of CYP2E1 by Essential Oils

CYP2E1 was tested with various essential oils to determine their inhibitory effects on the enzyme activity. Primarily the goal was to identify essential oils with strong inhibitory potency toward CYP2E1. Figure 12, shows several of the essential oils that were tested against the 2E1 enzyme. The data ranged from barely any inhibition to very potent inhibition, as seen with tarragon. Tarragon was selected out of the five essential oils that were tested because it demonstrated the greatest level of inhibition. The

main constituent found in tarragon is estragole; from this it was proposed that estragole might be the cause of inhibition of activity of CYP2E1.

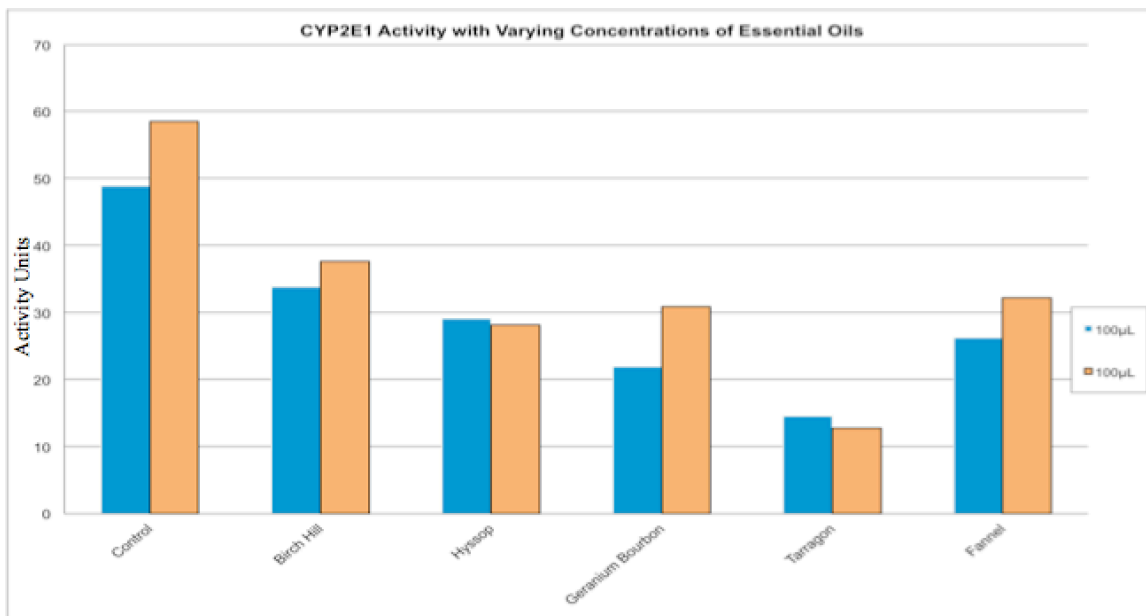


Figure 12. Screening Data for Essential Oils Inhibitory Affect on CYP2E1. Showing data for duplicates for each concentration of oil.

According to literature reports, basil oil also has a relatively high concentration of estragole present within it. From this information basil oil was also added to the project to determine its inhibitory effects on CYP2E1. Gas Chromatography Mass spectrometry (GCMS) analysis was done on both oils to confirm the presence of estragole, as seen in figure 13. Both oils along with pure estragole were tested with rat, rabbit, and human liver microsomes to determine their inhibitory effects on the P450 enzymes of each species.



Figure 13. GCMS Spectra Analysis of Tarragon and Basil. Chromatogram confirming the presence of estragole in both tarragon and basil Oil. Peak that appears at approximately 6.95 minutes is the estragole peak (estragole represented by the color blue, tarragon's estragole peak is represented by the color black, and basil's by the color pink), panel A shows the overall chromatogram and panel B shows a zoomed version of it.

3.1.2 Dose Response Studies

Initial screening experiments show that the CYP2E1 enzyme is being inhibited with increasing concentration of the oils. Both essential oils and estragole were initially prepared by diluting 5 μL pure oil (in the case of estragole, pure compound) into 100 mL of deionized water. Each of the oils and estragole were included in a reaction that had 50 μM *p*-nitrophenol. The concentration of the oils and estragole ranged from 0.0 $\mu\text{g/mL}$ to

23.00 $\mu\text{g/mL}$. Figure 14A-C, shows the percent activity of CYP2E1 remaining with the addition of varying concentration of inhibitor. Microsomes from all three species were used. In all three species, the basil oil assays required a higher concentration of inhibitor for the activity to decrease by over 50%; this could possibly be explained by the lower concentration of estragole present in basil oil. The tarragon oil has a higher concentration of estragole present, thus the data in figure 14A-C appear to support this idea (estragole is the inhibitory constituent in both basil and tarragon oil). The assay that had estragole and tarragon with the human and rabbit liver microsomes had a decrease in activity over 50% with the addition of 64 μM and 9.2 $\mu\text{g/mL}$ of inhibitor, respectively. Human and rabbit liver microsomes with basil oil required 13.8 $\mu\text{g/mL}$ to lose over 50% of its initial activity. The assay with estragole and tarragon that had rat liver microsomes had a decrease in activity over 50% with the addition of only 32 μM and 4.6 $\mu\text{g/mL}$ of inhibitor respectively. Basil oil with the rat liver microsomes required 9.2 $\mu\text{g/mL}$ to lose over 50% of its initial activity (possibly due to the lower concentration of estragole). In general, the rat liver microsomes appeared to be more sensitive to the inhibitors compared with the human and rabbit. Table 2 shows the IC_{50} values calculated for each inhibitor with all 3 species. It should be noted that the concentrations of estragole of 64 mM and 32 mM correspond to 9.2 $\mu\text{g/mL}$ and 4.6 $\mu\text{g/mL}$, respectively. Given that the overall content of the tarragon is >80% estragole, this is fully consistent with our assertion that estragole is the active component of the tarragon oil.

Table 2. IC₅₀ Values for Inhibitors with CYP2E1.

	Tarragon	Basil	Estragole
Human Liver CYP2E1- IC₅₀	9.2 µg/mL	13.8 µg/mL	64 µM
Rabbit Liver CYP2E1- IC₅₀	9.2 µg/mL	13.8 µg/mL	64 µM
Rat Liver CYP2E1- IC₅₀	4.6 µg/mL	9.2 µg/mL	32 µM

14-A.

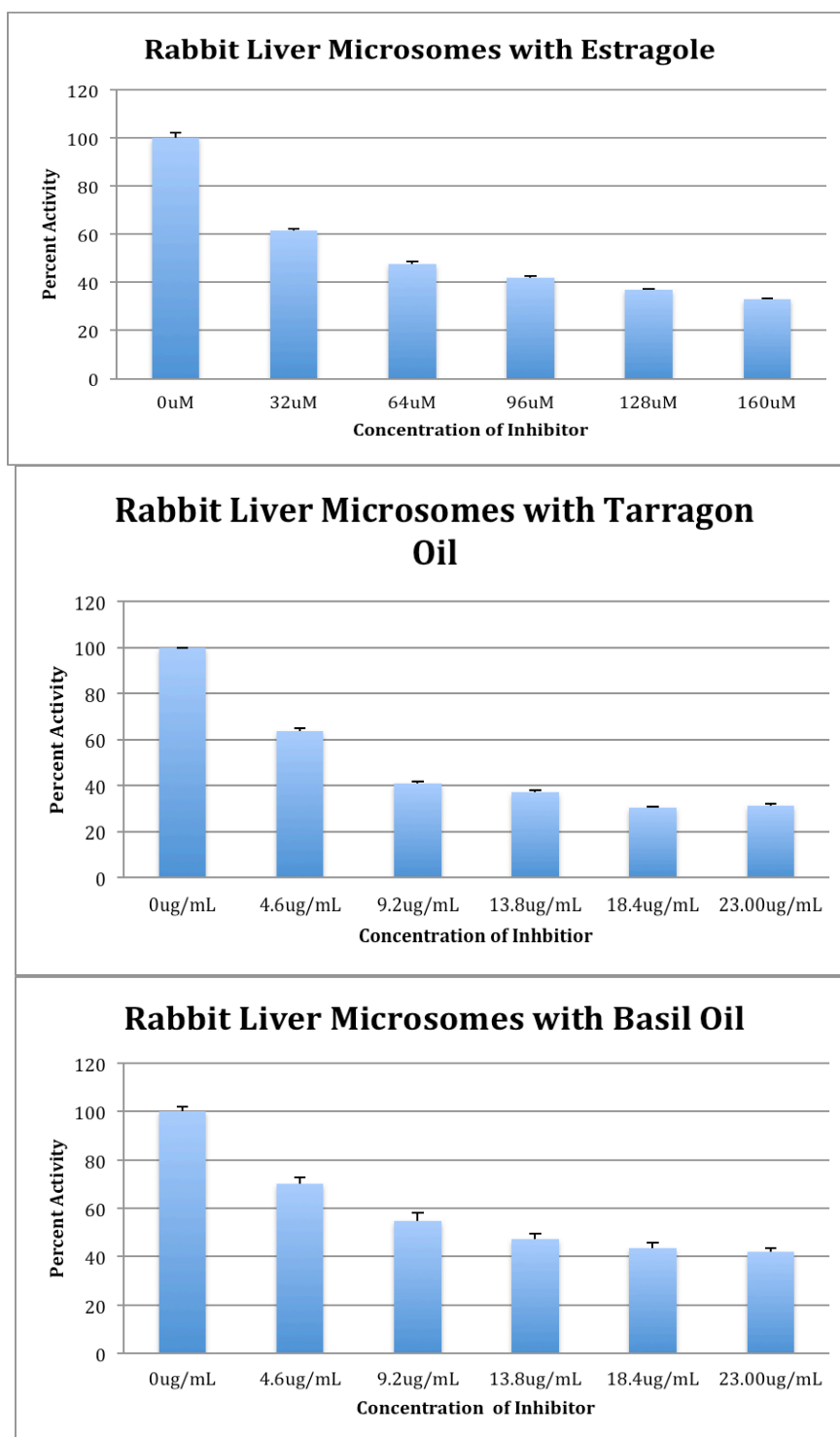


Figure 14-A. Dose Response Studies with CYP2E1-Rabbit.

14-B.

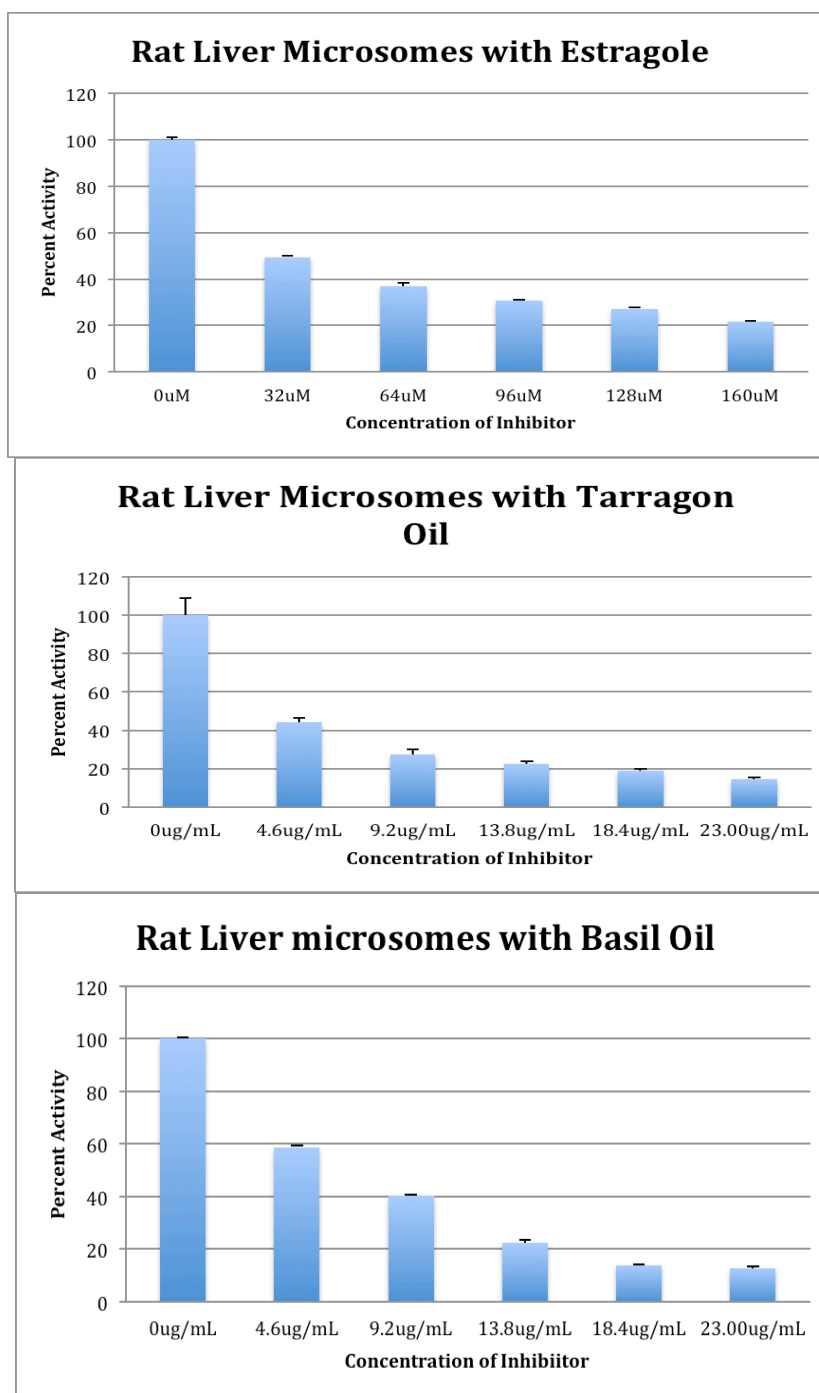


Figure 14-B. Dose Response Studies with CYP2E1-Rat.

14-C.

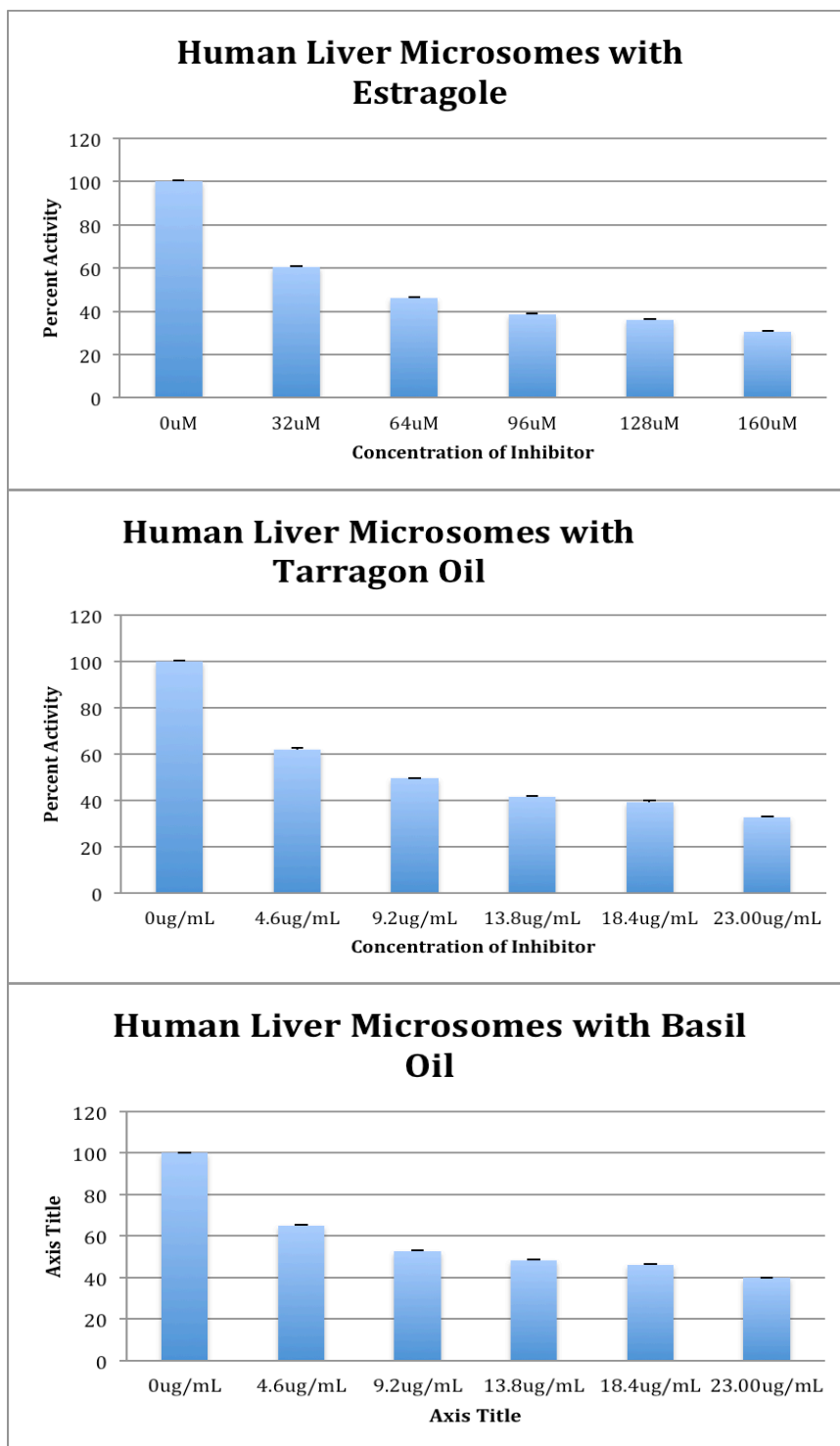


Figure 14-C. Dose Response Studies with CYP2E1-Human.

3.1.3 Michaelis-Menten Studies-CYP2E1

The Michaelis-Menten model of enzyme kinetic and inhibition was used to obtain a more accurate representation of the inhibitory effects of the essential oils on CYP2E1. The kinetics study was carried out with different concentrations of the substrate, *p*-NP, ranging from 10 μ M-100 μ M in the presence and absence of the inhibitor. These reactions were carried out in duplicates to confirm the results were reproducible. The concentration of inhibitors used was determined based on previous studies carried out where the enzyme inhibition of ~50% was observed. Human, rabbit, and rat liver microsomes were used to generate Michaelis-Menten graphs. When compounds inhibit an enzyme there can be a change in the V_{\max} and/or K_m of the substrate reaction. From determining the K_m^{app} and V_{\max}^{app} the mode of inhibition by the inhibitor can be determined. Determining the dissociation constant, K_i , is important because it defines the amount of inhibitor needed to induce inhibition of the enzyme, and is a general measure of the potency of the inhibitor.

Figure 15 shows the Michaelis-Menten graph for the oxidation of *p*-nitrophenol by human liver microsomes in the presence and absence of inhibitor. The V_{\max} for the reaction with no inhibitor was 468.73 activity units (defined as the peak area for the product formed in a 30 min reaction). When tarragon, basil, and estragole were added to the reaction, there was a drastic decrease in V_{\max}^{app} . The V_{\max}^{app} for the inhibitors were as follows: tarragon, 297 AU, basil, 312 AU, and estragole, 168 AU. The K_m values for the reaction varied only slightly: with no inhibitor in reaction K_m : 37, tarragon K_m : 60, basil K_m : 49, and estragole K_m : 47. When V_{\max} is reduced and K_m remains the same, this

suggests that the inhibition is non-competitive. Using equation 2, the K_I values were calculated for each inhibitor, K_I values are as followed, tarragon: 10.0 $\mu\text{g/mL}$, basil: 11.5 $\mu\text{g/mL}$, and estragole: 22.4 μM .

MM Studies-Human Liver Microsome-CYP2E1

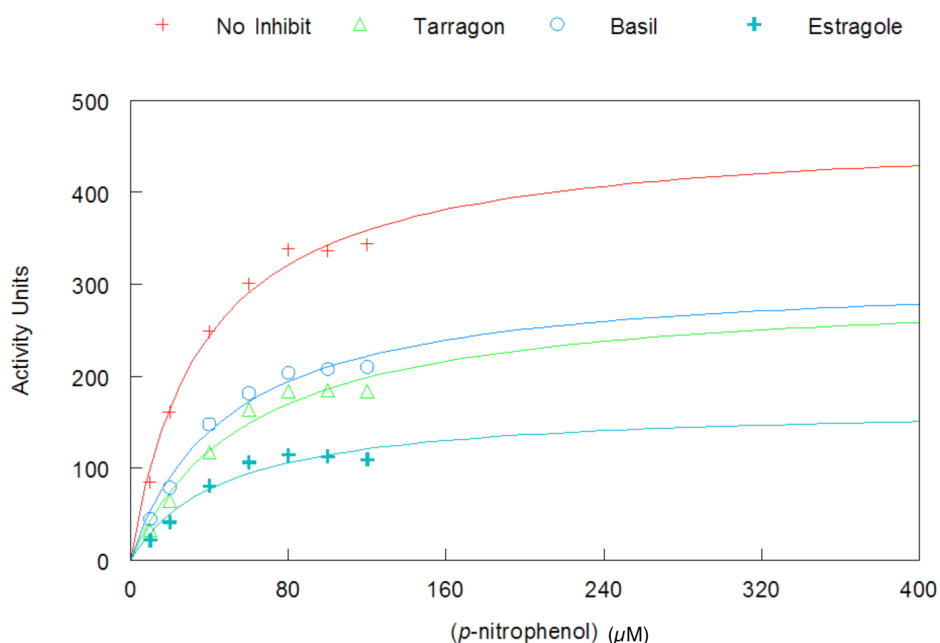


Figure 15. Michaelis-Menten Plot for CYP2E1-Human Liver. Results for Human liver microsomes with the presence and absence of inhibitor.

Michaelis-Menten kinetic studies were carried out with rat liver microsomes with the presence and absence of inhibitor, which can be seen in figure 16. V_{\max} for the reaction without the addition of inhibitor was 346 AU. With the addition of tarragon, basil, and estragole to the reaction, there was a decrease in V_{\max}^{app} . The V_{\max}^{app} for the inhibitors was as follows: tarragon: 283AU, basil: 291 AU, and estragole: 271 AU. The

K_m values of the reaction stayed in approximately the same range, with no inhibitor in reaction the value of K_m was: 48 mM, tarragon K_m : 52 mM, basil K_m : 51 mM , and estragole K_m : 52 mM. Using equation 2, the K_I values were calculated for each inhibitor, K_I values are as followed, tarragon: 26 μ g/mL, basil: 30 μ g/mL, and estragole: 143 μ M. With the V_{max} changing considerably and the K_m remaining reasonably constant, a non-competitive form of inhibition was inferred. Interestingly, the results from the Michaelis-Menten analysis suggest that the inhibitor is more potent with human liver CYP2E1 as compared to rat. Although this appears to conflict with the IC_{50} values reported previously, it should be noted that the human 2E1 has a lower K_m for the substrate, so using the same concentration of substrate for the IC_{50} determinations could give the appearance of a more potent binding to the rat enzyme in those experiments. As a general rule, K_I is a more accurate measure of potency than IC_{50} .

M.M Studies-Rat Liver Microsomes-CYP2E1

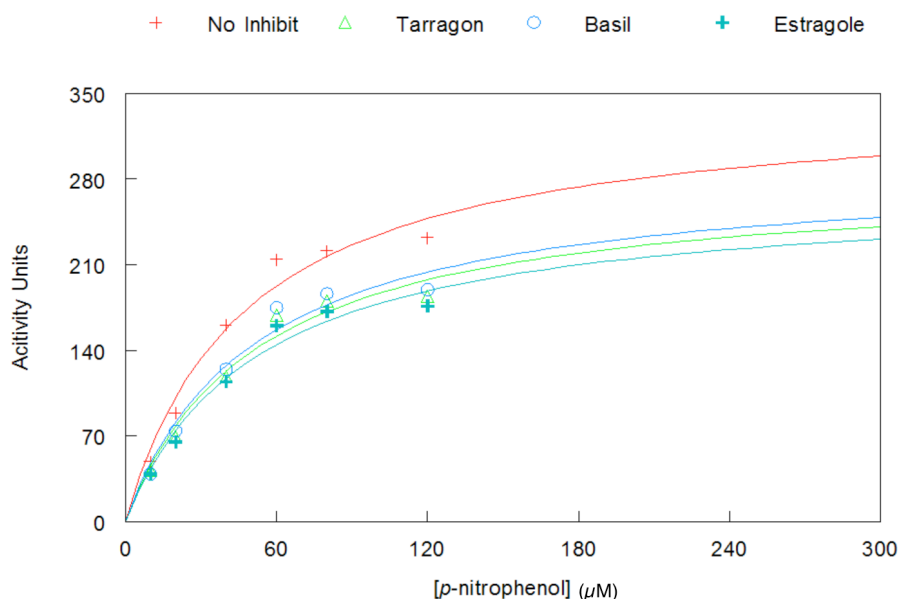


Figure 16. Michaelis-Menten Plot for CYP2E1-Rat Liver. Results for rat liver microsomes with the presence and absence of inhibitor.

Michaelis-Menten kinetic studies were carried out with Rabbit liver CYP2E1 enzymes. The presence and absence of inhibitor were both studied in order to determine the type of inhibition occurring, results for this could be seen in figure 17. The V_{\max} for the reaction without inhibitor was 94 AU. When the inhibitor was added to the reaction there was a decrease in enzymatic activity. The V_{\max}^{app} for the inhibitors was as follows, tarragon: 72 AU, basil: 80 AU, and estragole: 69 AU. The K_m values of the reaction stayed in approximately the same range, with no inhibitor in reaction the value of K_m was: 48 mM, tarragon K_m : 58 mM, basil K_m : 56 mM, and estragole K_m : 55 mM. Using equation 2, the K_i values were calculated for each inhibitor, K_i values were as follows, tarragon: 19 μg/mL, basil: 32 μg/mL, and estragole: 108 μM. With the V_{\max} changing, not

as drastically as was seen with the human and rat liver P450, and K_m staying constant, this suggests non-competitive form of inhibition (for comparative analysis, V_{max} , K_m , and K_i values of all 3 species can be found in table 4). Again, here the K_i values are higher than the ones with human liver, suggesting that the inhibitor is more potent in human verses rabbit. Comparing it to rat liver, then the inhibitors are less potent with rat verses rabbit. Again, the higher value of the K_m for the substrate observed with the rabbit may explain why in the Michaelis-Menten study, the apparent potency of the inhibitors toward the rabbit P450 is lower than that of the rat and human enzymes.

M.M Studies-Rabbit Liver Microsomes-CYP2E1

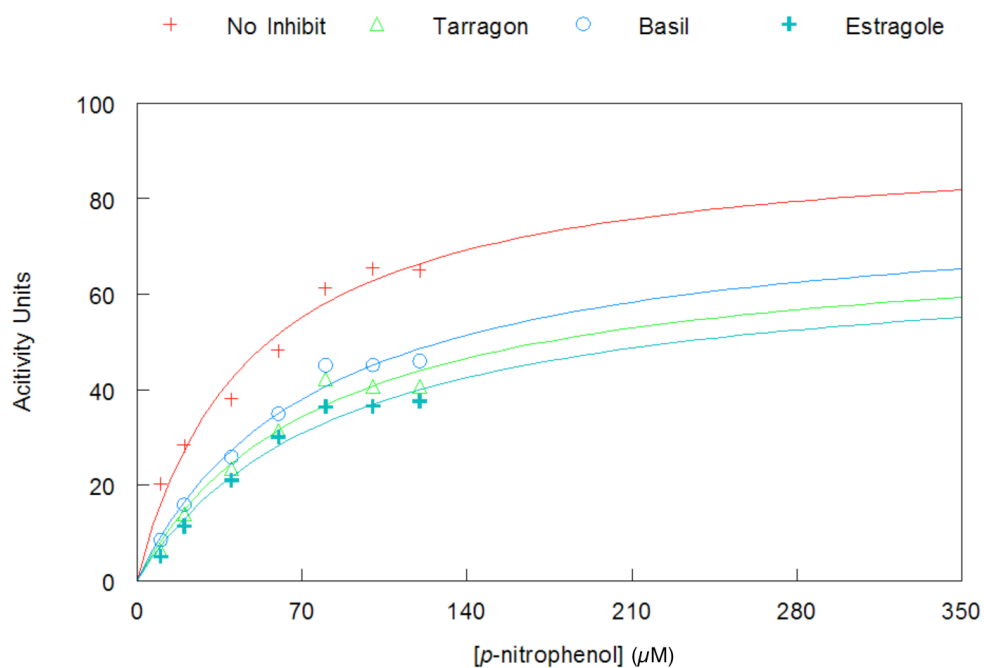


Figure 17. Michaelis-Menten Plot for CYP2E1-Rabbit Liver. Results for rabbit liver microsomes with the presence and absence of the inhibitors.

Table 3. Comparative Analysis Showing Values of V_{max} , K_m , and K_i -CYP2E1.

Human	V_{max} (AU)	K_m (uM)	K_i
No Inhibitor	469 ± 26	36.75 ± 5.72	-
Tarragon	297 ± 39	59.96 ± 17.80	9.96 µg/mL
Basil	313 ± 27	49.03 ± 10.25	11.50 µg/mL
Estragole	168 ± 23	47.14 ± 16.19	3.21 µg/mL
Rat	V_{max} (AU)	K_m (uM)	K_i
No Inhibitor	347 ± 44	47.99 ± 14.09	-
Tarragon	283 ± 38	52.01 ± 15.58	25.62 µg/mL
Basil	290 ± 39	51.16 ± 15.63	29.99 µg/mL
Estragole	270 ± 36	52.20 ± 15.51	20.54 µg/mL
Rabbit	V_{max} (AU)	K_m (uM)	K_i
No Inhibitor	94 ± 9	48.42 ± 11.37	-
Tarragon	73 ± 11	58.11 ± 22.76	19.48 µg/mL
Basil	80 ± 9	56.16 ± 17.55	31.60 µg/mL
Estragole	69 ± 11	54.50 ± 27.07	15.90 µg/mL

3.1.4 Two-Stage Reversibility Study Results

Research on tarragon and basil showed that their major constituent estragole had a terminal olefin present, which may be a suicide substrate for the enzyme through irreversible binding and activation at the active site. To determine if the inhibitor was reversible or irreversible, a two-stage reversibility study was done. Going by the results displayed in Figures 18 A-B and 19 A-B, the reaction appeared to be reversible. This is because all the reactions showed almost equal amounts of activity, regardless of how the samples were pre-treated the changing of parameters had no overall affects on enzyme activity. For example, a mechanism-based inhibitor would be expected to inactivate the

enzyme when NADPH and inhibitor were pre-incubated with the enzyme, as was the case in figure 18A-B To confirm this, time-dependent studies were carried out using tarragon, basil, and estragole.

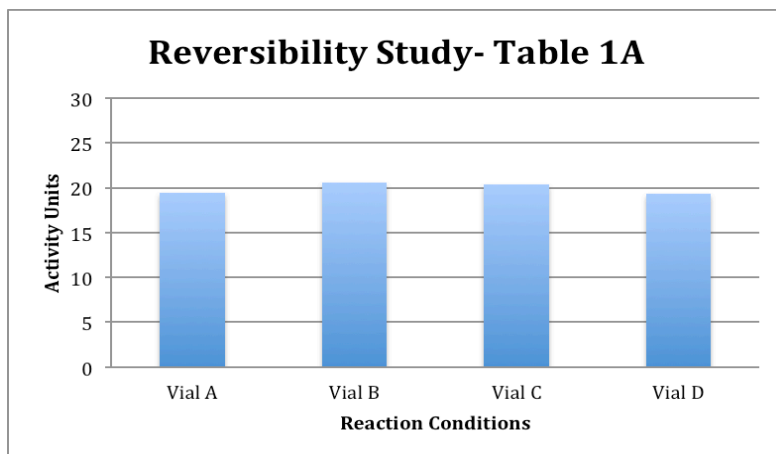


Figure 18-A. Reversibility Study-Table 1 A Graph-First Parameter. Graph displays the measured activity of each vial from the reversibility study (first reaction as mentioned in-text above).

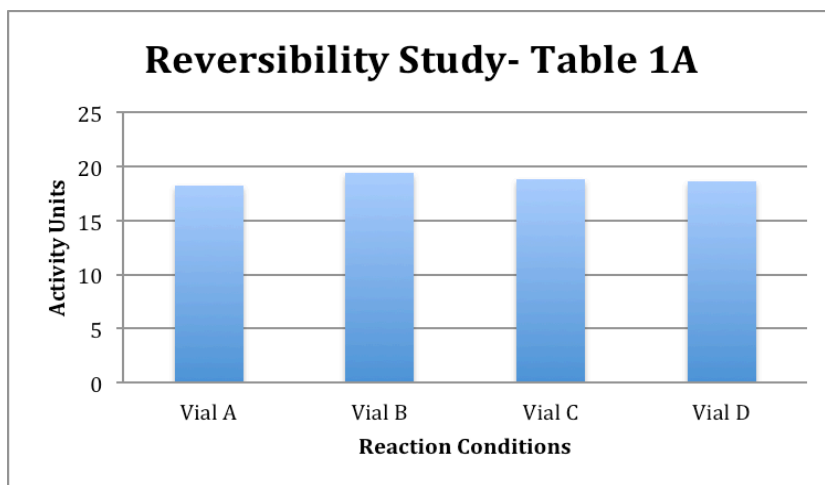


Figure 18-B. Reversibility Study-Table 1 A Graph-Second Parameter. Graph displays the measured activity of each vial from the reversibility study (second reaction as mentioned in-text above).

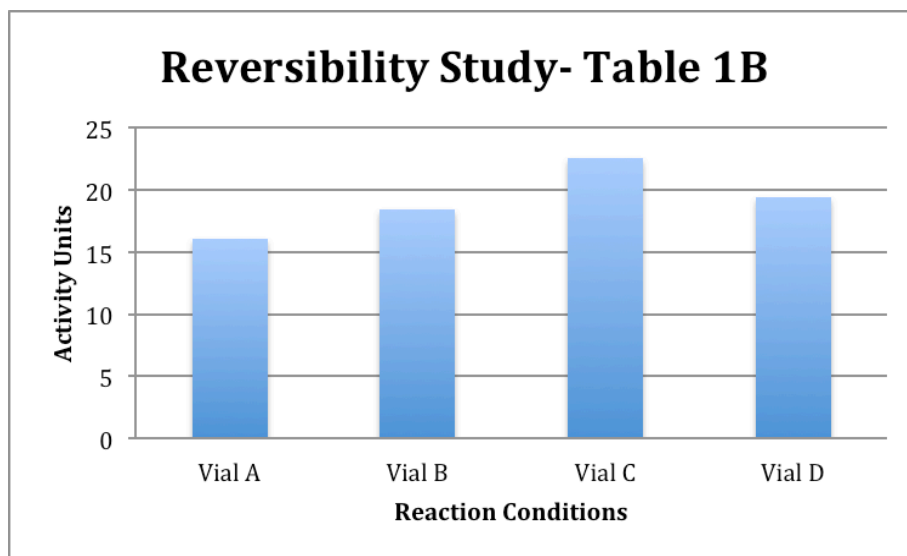


Figure 19-A. Reversibility Study-Table 1 B Graph-First Parameter. Graph displays the measured activity of each vial from the reversibility study (first reaction as mentioned in-text above).

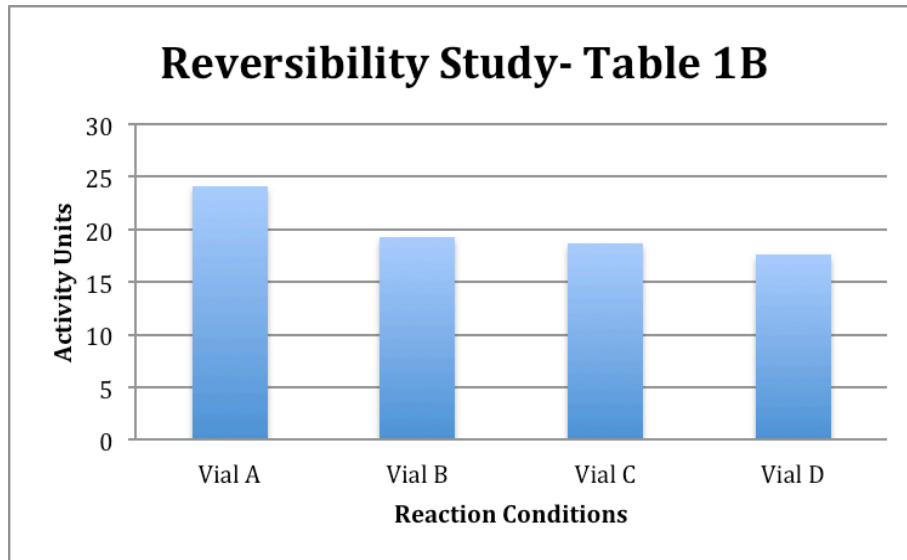


Figure 19-B. Reversibility Study-Table 1 B Graph-Second Parameter. Graph displays the measured activity of each vial from the reversibility study (second reaction as mentioned in-text above).

3.1.5 Time-Dependent Study Results

Time-dependent studies were carried out to confirm that no irreversible inactivation of the enzyme by the essential oils occurred. The reaction was carried out over the course of 120 minutes with reactions being quenched at 10, 20, 40, 60, 80, and 120 minutes. This was done to monitor enzyme inactivation via the inhibitor over a course of time. If the inhibitors were irreversible then one should observed activity loss by the enzyme at a faster rate in the presence of inhibitor than in the absence. Complicating the experiment was the fact that the control reaction also lost activity over time, so taking the ratio of the product formed in the absence vs the presence of inhibitor was used as a measure of inactivation. In other words, activity loss would be much more rapid in the presence of an irreversible inhibitor. However, if the inhibitors were reversible then these ratios should remain relatively constant over duration of the reaction. Figure 20A-C shows the results of these studies. Results show that over time there is no excess inactivation of the enzyme suggesting that the inhibitor is a reversible inhibitor.

CYP2E1-Reversibility Study-Human Liver

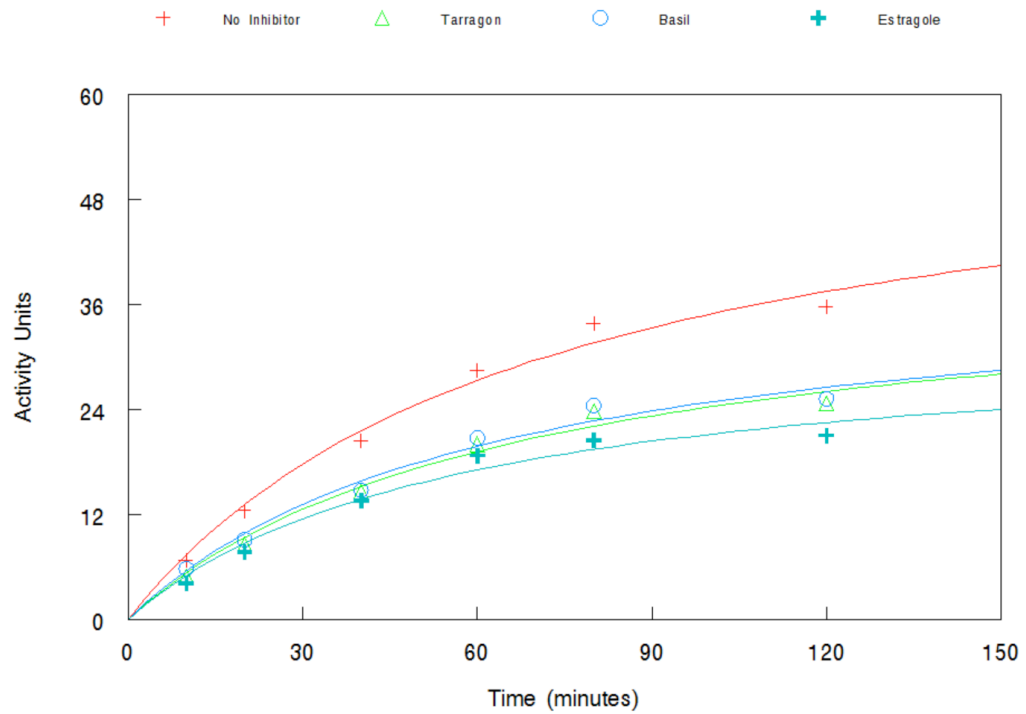


Figure 20-A. Time-Dependent Studies Graph-CYP2E1-Human Liver. Time-dependent studies carried out with both essential oils and estragole on human liver microsomes.

CYP2E1-Reversibility Study-Rat Liver

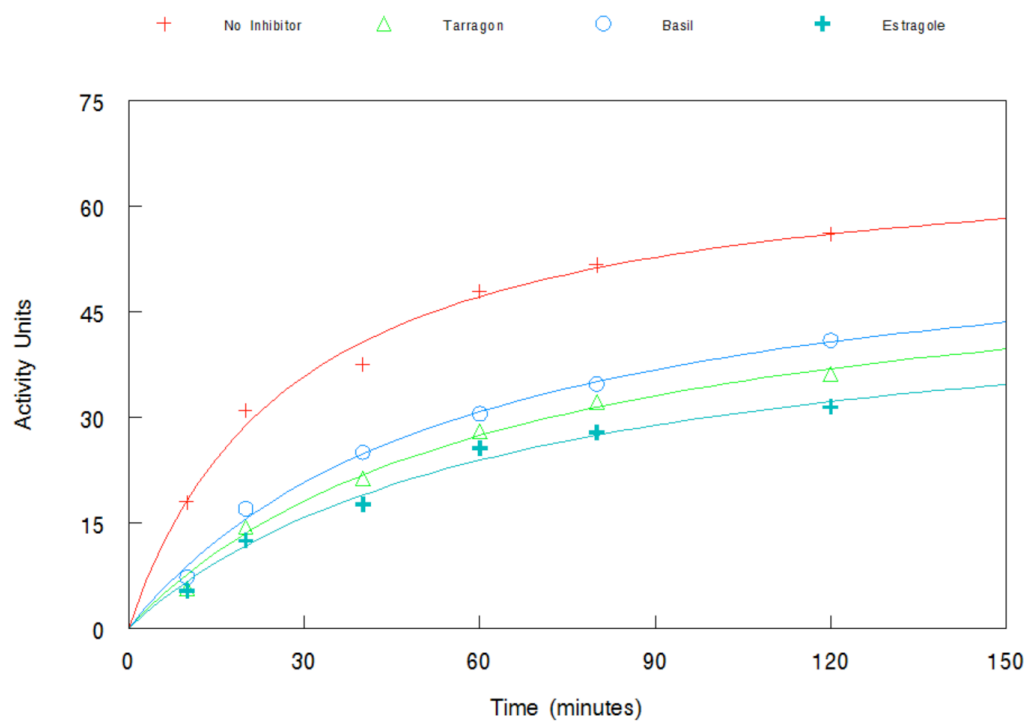


Figure 20-B. Time-Dependent Studies Graph-CYP2E1-Rat Liver. Time-dependent studies with rat liver microsomes and the effects of inhibitors tarragon, basil, and estragole.

CYP2E1-Reversibility Study-Rabbit Liver

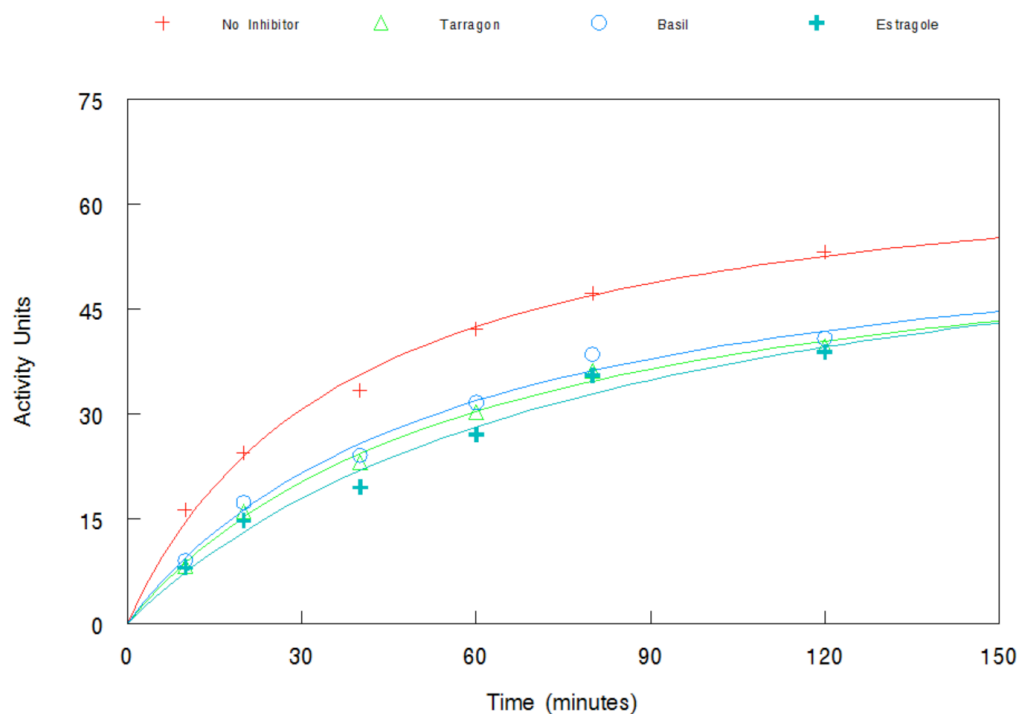


Figure 20-C. Time-Dependent Studies Graph-CYP2E1-Rabbit Liver. Time-dependent studies with rabbit liver microsomes and the essential oils with there major constituent estragole.

3.2.0 CYP2A6 Enzyme Activity

To setup the bioassay involving CYP2A6 several parameters were tested in order to determine optimum conditions to carry out the assay. This process involved testing liver microsomes from human and rabbit; (rats don't contain the enzyme CYP2A6), with varying concentrations of enzyme, incubation time, and substrate concentration. The assay was prepared using conditions mentioned previously. Figure 21 shows how varying concentration of microsomes were tested with the substrate, coumarin, to show its effects on the formation of 7-hydroxycoumarin. The results were expected, an approximately linear increase in product formation was observed with increase in microsomal concentration. Based on the test results from these experiments it was determined that 20 μ L of the rabbit liver microsomes and 5 μ L of the human liver microsomes were chosen for the assays involving CYP2A6.

Another component tested for the assay was the affect of time on product formation. As reaction time increases this should also increase the amount of product form, assuming no inactivation happens. Figure 22 shows an increase in product formation with CYP2A6 as reaction duration is increased. The increase was only linear for a short period of time, however, so for inhibition studies, durations of 30 min or less were utilized.

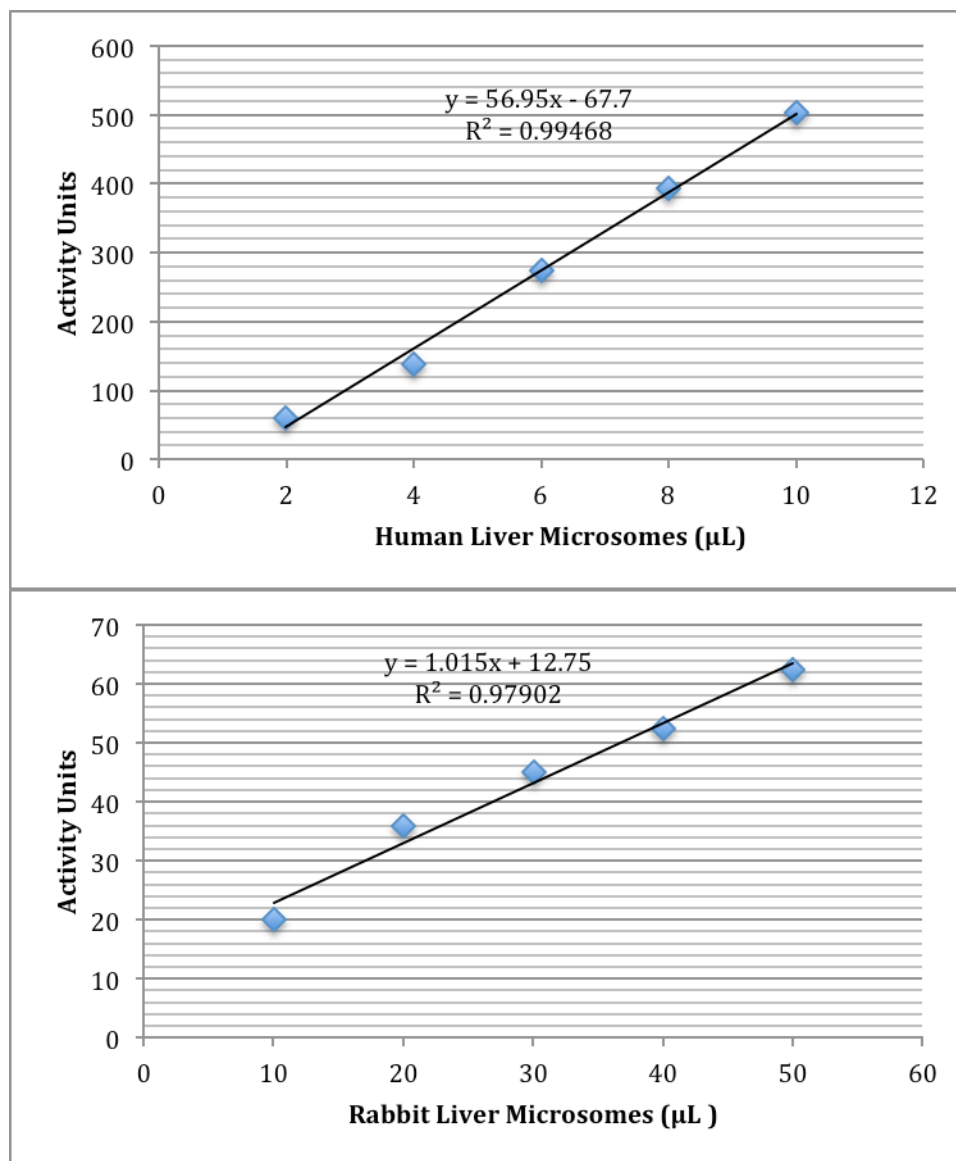


Figure 21. Results for Variation in Microsomal Concentration-CYP2A6. Effects of varying concentration of microsome on formation of 7-hydroxycoumarin. These results determined amount of micsrome was used in each respective assay.

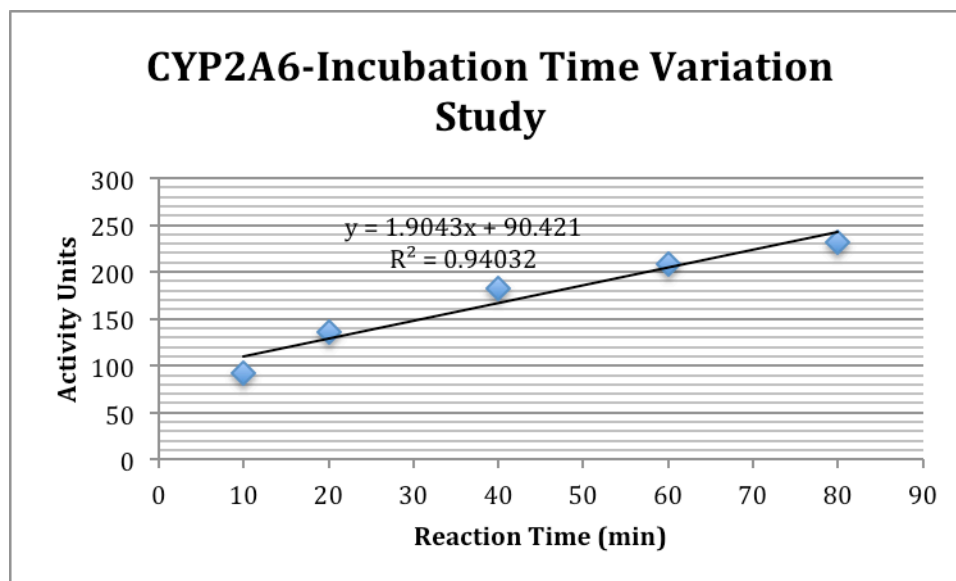


Figure 22. Results for Variation in Incubation Time-CYP2A6. Graph showing the effect of time on reaction rate, as time increases so does product formation. From this study it was determined that the appropriate time for the bioassay involving CYP2A6 will be 30 minutes.

3.2.1 Dose Response Studies

CYP2A6 has a small hydrophobic active site like its counterpart CYP2E1. Initial screening experiment showed inhibition of CYP2A6 with increasing concentration of the essential oils. The setup for these dose response study involved taking 5 μ L of tarragon oil, basil oil, and estragole and diluting it in 100 mL of deionized water. The substrate concentration used for this study was 5 μ M coumarin. The concentration of the oils ranged from 0.0 μ g/mL to 23.00 μ g/mL, and for estragole it was 0 μ M to 160 μ M. The results of these studies can be seen in figure 23A-B. The inhibitors were tested with both human and rabbit liver microsomes. The results showed that the oils and estragole had a higher potency toward CYP2A6 as compared to CYP2E1. The basil oil required a higher concentration for the activity to be inhibited by 50%, this again is explained by the fact

there is a lower concentration of estragole in basil oil, thus requiring a higher concentration of it. Tarragon oil has a higher concentration of estragole thus not requiring as much to inhibit the enzymatic activity by 50%. Estragole itself was potent on both species, results of the experiments show that it dramatically decreased enzyme activity. This supports the idea that it is the major constituent in both oils and is responsible for the enzyme activity being inhibited. With rabbit, 50% inhibition with tarragon occurred with 9.2 µg/mL, with basil at 13.8 µg/mL, and with estragole at 64µM. The potency of the inhibitors were increased with the human liver CYP2A6. With human, 50% inhibition with tarragon occurred at 4.6 µg/mL, with basil at 9.2 µg/mL, and estragole with 32 µM. The human liver microsomes were more sensitive than the rabbit liver microsomes. Table 3 shows the IC₅₀ values for each respective inhibitor with both species.

Table 4. IC₅₀ Values for Inhibitors with CYP2A6.

	Tarragon	Basil	Estragole
Human Liver CYP2A6- IC₅₀	4.6 µg/mL	9.2 µg/mL	32 µM
Rabbit Liver CYP2A6- IC₅₀	9.2 µg/mL	13.8 µg/mL	64 µM

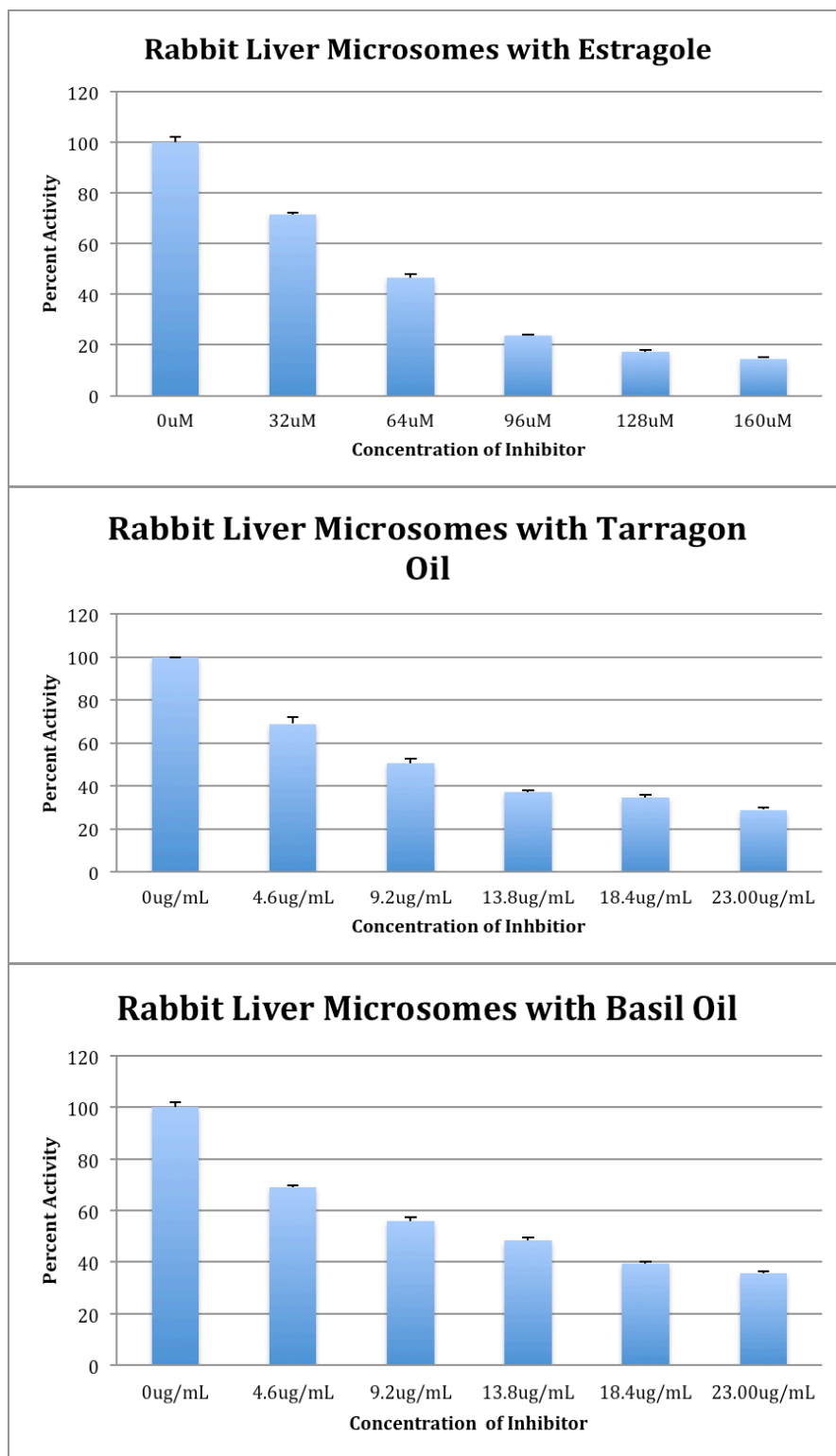


Figure 23-A. Dose Response Studies with CYP2A6-Rabbit Liver.

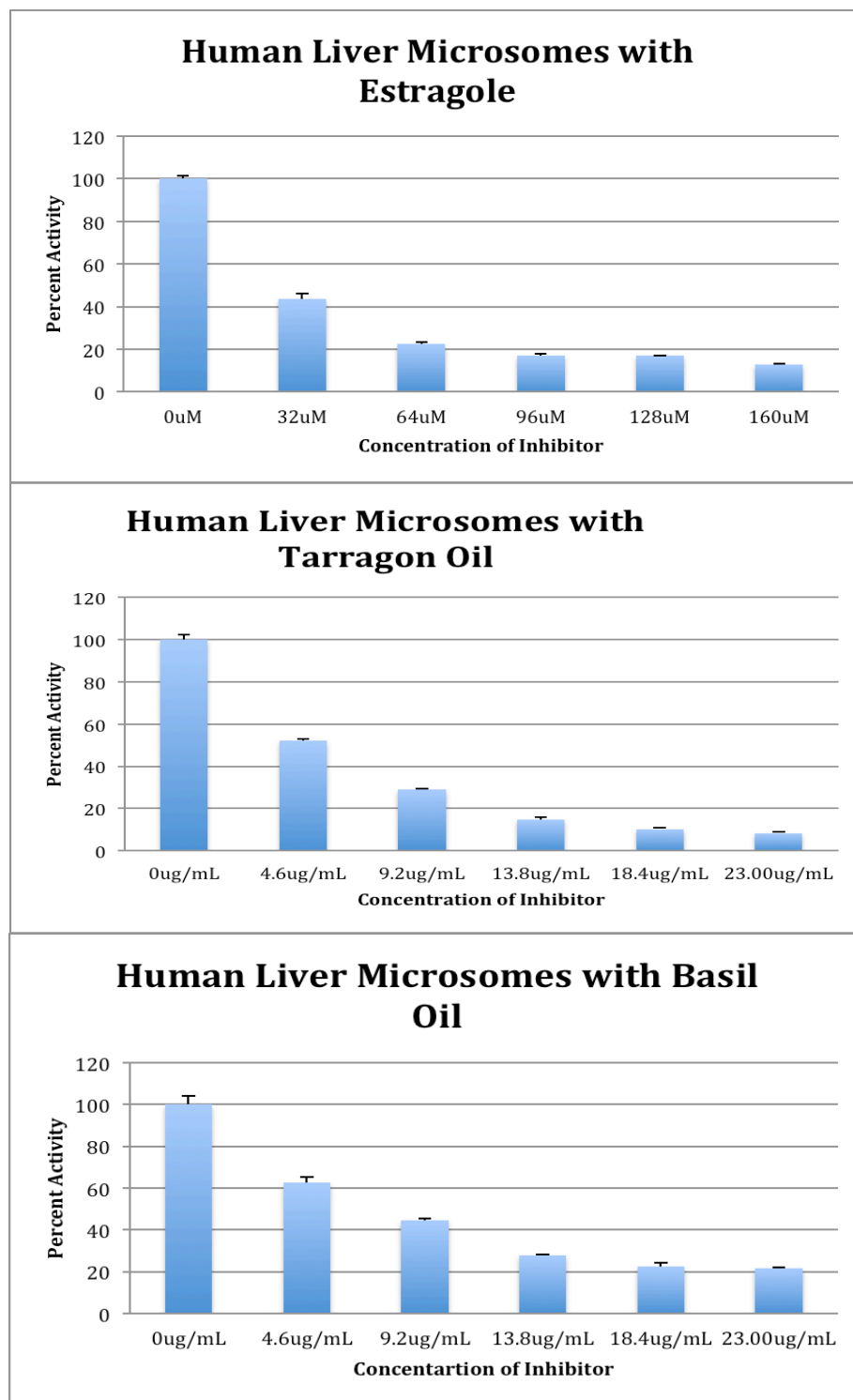


Figure 23-B. Dose Response Studies with CYP2A6-Human Liver.

3.2.2 Michaelis-Menten Studies-CYP2A6

To study the kinetics of the enzyme, Michaelis-Menten studies were carried out. This model was used to find the mode of inhibition by the inhibitors on the enzyme, CYP2A6. The kinetic study was carried out with different concentrations of the substrate, coumarin, ranging from 1 μ M-10 μ M in the presence and absence of the inhibitor. The concentration of inhibitors used was determined based on previous studies carried out where the enzyme activity is inhibited by 50%. The reactions were carried out in duplicates in order to confirm the results were reproducible. The study was carried out with both human and rabbit liver microsomes with tarragon oil, basil oil, and estragole. The data generated with the Michaelis-Menten graph was used to determine V_{\max} , V_{\max}^{app} , K_m and K_m^{app} and from this information K_i was calculated.

Figure 24 shows the Michaelis-Menten graph of *human* liver microsomes with the presence and absence of inhibitor. V_{\max} for the reaction without the inhibitor was 103AU. When the essential oils and estragole was added to the reaction, there was a decrease in V_{\max}^{app} . The V_{\max}^{app} for the inhibitors were as follows: tarragon, 47 AU, basil, 46 AU, and estragole, 43 AU. The K_m values of the reaction stayed in approximately the same range, with no inhibitor in reaction K_m : 6.5 mM, tarragon K_m : 5.0 mM, basil K_m : 3.7 mM, and estragole K_m : 5.0 mM. V_{\max} being reduced in the reaction and K_m remaining in the same suggests that the inhibition is non-competitive. Using equation 2, the K_i values were calculated for each inhibitor, K_i values were calculated as follows, tarragon: 4.8 μ g/mL, basil: 4.6 μ g/mL, and estragole: 27.5 μ M.

M.M Studies-Human Liver Microsome-CYP2A6

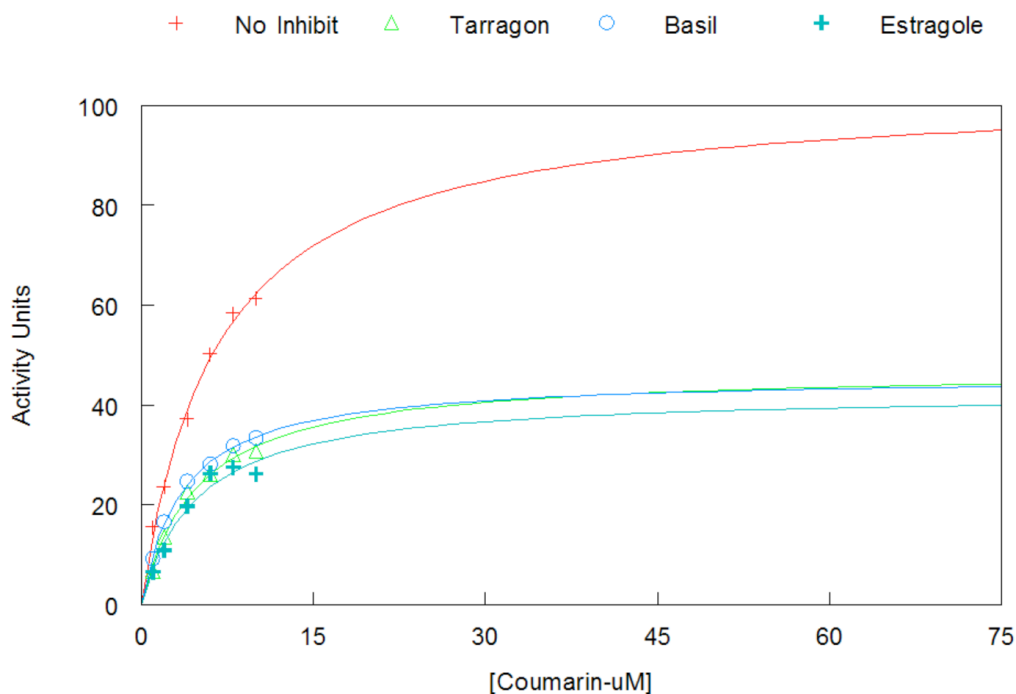


Figure 24. Michaelis-Menten Plot for CYP2A6-Human Liver. The graph is showing data of human liver enzyme with the presence and absence of inhibitor.

Michaelis-Menten studies were carried out with rabbit liver CYP2A6. Figure 25 shows the results of these studies. The V_{\max} for the reaction with no inhibitor was 113.24. With the addition of tarragon, basil, and estragole, the V_{\max}^{app} decreased. The values for V_{\max}^{app} are as follows: tarragon: 72 AU, basil: 74 AU, and estragole: 63 AU. The K_m values of the reaction stayed in approximately the same range, with no inhibitor in reaction the value of K_m was: 6.0 μM , tarragon K_m : 7.1 μM , basil K_m : 5.9 μM , and estragole K_m : 8.3. μM Using equation 2, the K_I values were calculated for each inhibitor, K_I values are as followed, tarragon: 9.9 $\mu\text{g/mL}$, basil: 10.8 $\mu\text{g/mL}$, and estragole: 49.3

μM . Again here the V_{\max} is changing, and the K_m values are staying within the same range suggesting that it is a non-competitive inhibition. The K_i values are higher than the human CYP2A6 values, suggesting that the inhibitor is less potent on *rabbits* versus *humans* (for comparative analysis, V_{\max} , K_m , and K_i values of both species can be found in table 5).

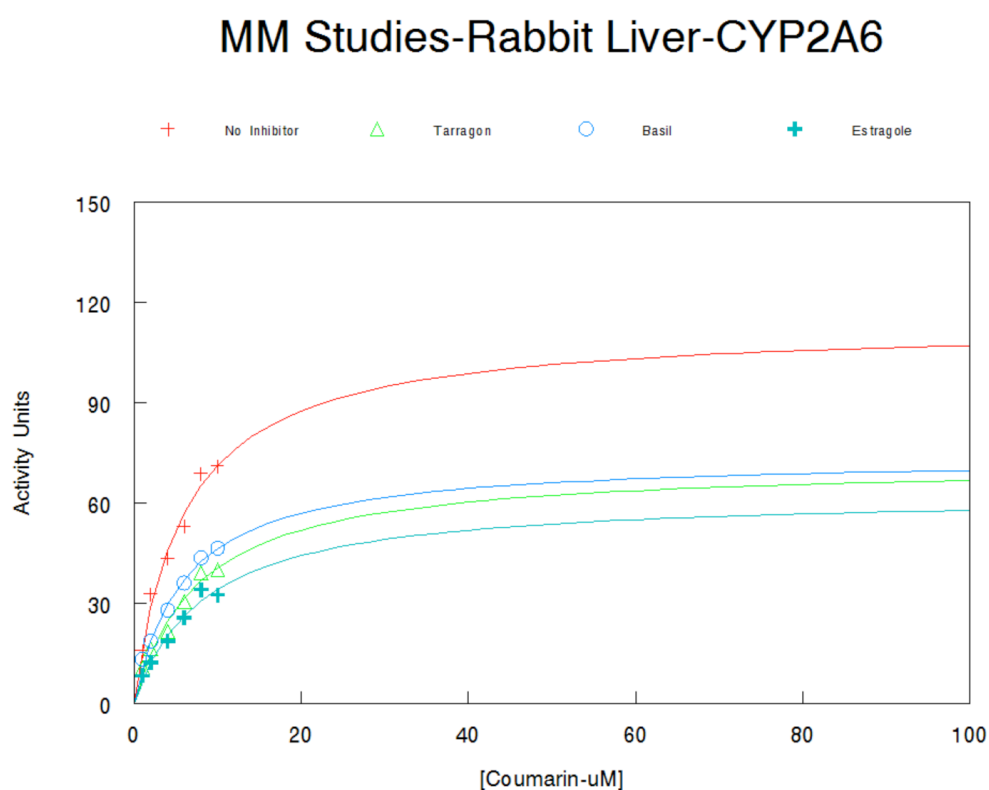


Figure 25. Michaelis-Menten Plot for CYP2A6-Rabbit Liver. Graph showing results of rabbit liver microsomes with the presence and absence of tarragon, basil, and estragole.

Table 5. Comparative Analysis Showing Values of V_{\max} , K_m , and K_i -CYP2A6.

Human	V_{\max} (AU)	K_m (uM)	K_i
No Inhibitor	103 ± 17	6.54 ± 2.94	-
Tarragon	47 ± 3	5.00 ± 1.00	4.80 µg/mL
Basil	46 ± 3	4.00 ± 1.00	4.58 µg/mL
Estragole	46 ± 5	5.00 ± 1.50	4.10 µg/mL
Rabbit	V_{\max} (AU)	K_m (uM)	K_i
No Inhibitor	113 ± 14	6.00 ± 1.53	-
Tarragon	72 ± 12	7.10 ± 2.60	9.94 µg/mL
Basil	74 ± 7	5.96 ± 1.16	10.80 µg/mL
Estragole	62 ± 9	8.28 ± 2.26	7.28 µg/mL

3.2.3 Time Dependent Study Results

To determine the mode of inhibition, time-dependent studies were performed with the essential oils. Figure 26A-B shows the results of these studies. The reaction was carried out over the course of 80 minutes with the reactions being quenched at 10, 20, 40, 60, and 80 minutes. This reaction process was done to determine if there was enzyme inactivation by the inhibitor over the course of the run of the reaction. If the inhibitor is irreversibly binding to the enzyme then, as discussed in the previous section, an increase in the rate of activity loss should be observed in the presence of the inhibitor. If the inhibitor binding to the enzyme is reversible then the rate at which activity loss occurs should be comparable with or without inhibitor, as measured by the ratio of product formation without/with inhibitor at each time point. The results of this study show that the inhibitor appears to be reversible because the inhibitor does not influence the rate at which activity decreases over time.

CYP2A6-Reversibility Study-Human Liver

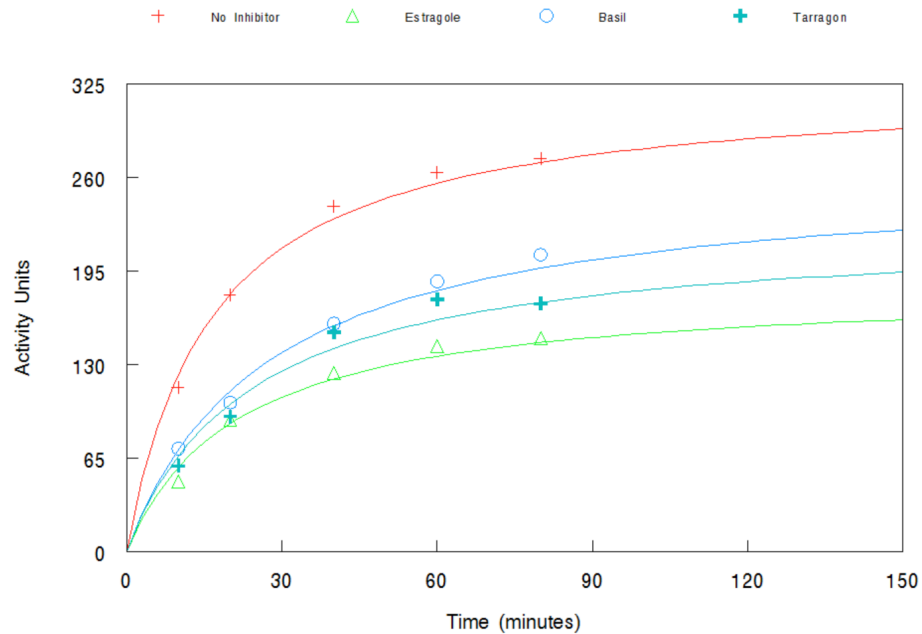


Figure 26-A. Time-Dependent Studies Graph-CYP2A6-Human Liver. Time-Dependent studies graph with human liver microsomes with the absence and presence of inhibitor.

CYP2A6-Reversibility Study-Rabbit Liver

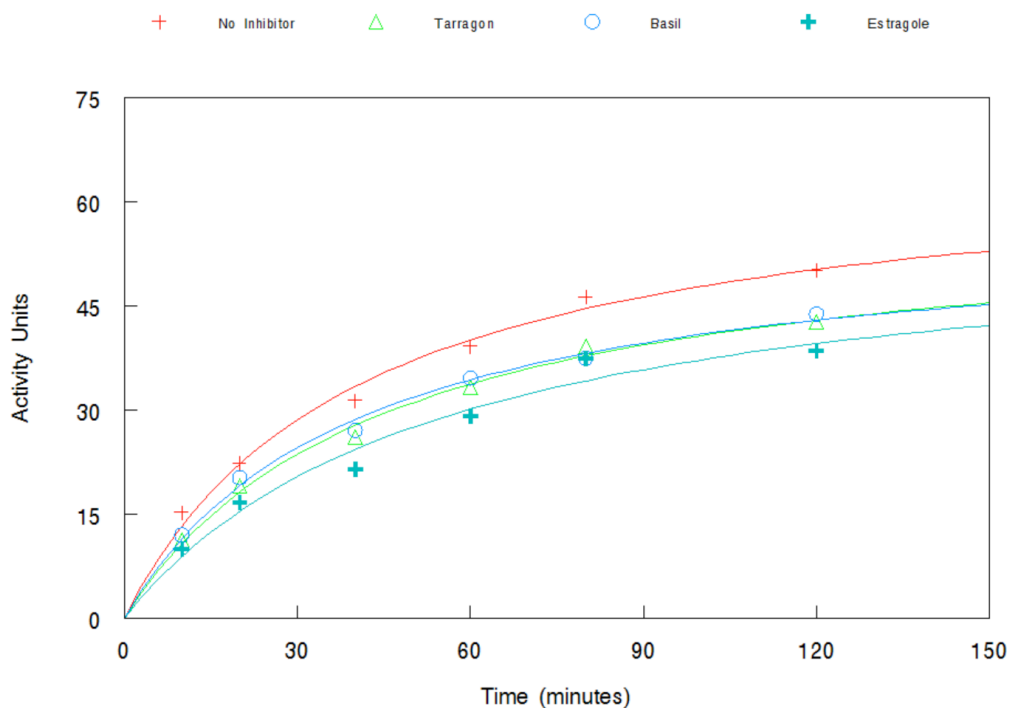


Figure 26-B. Time-Dependent Studies Graph-CYP2A6-Rabbit Liver. Time-dependent studies with rabbit liver microsomes and the impact of the inhibitor overtime.

3.3.0 Supersome Studies-CYP2E1 and CYP2A6

To confirm the results of the study, selectively expressed CYP P450 supersomes were used. CYP2A6 and CYP2E1 supersomes were used in a dose-response and time-dependent studies. The overall reaction setup for both experiments is the same as mentioned above for time-dependent and dose response experiments using the human liver samples. The results of the dose response study with both enzymes can be seen in figure 27A-B. The results are similar to the dose response studies with both enzymes in

human, rabbit, and rat. In other words, pure estragole appeared to be most potent followed by tarragon and then basil oils.

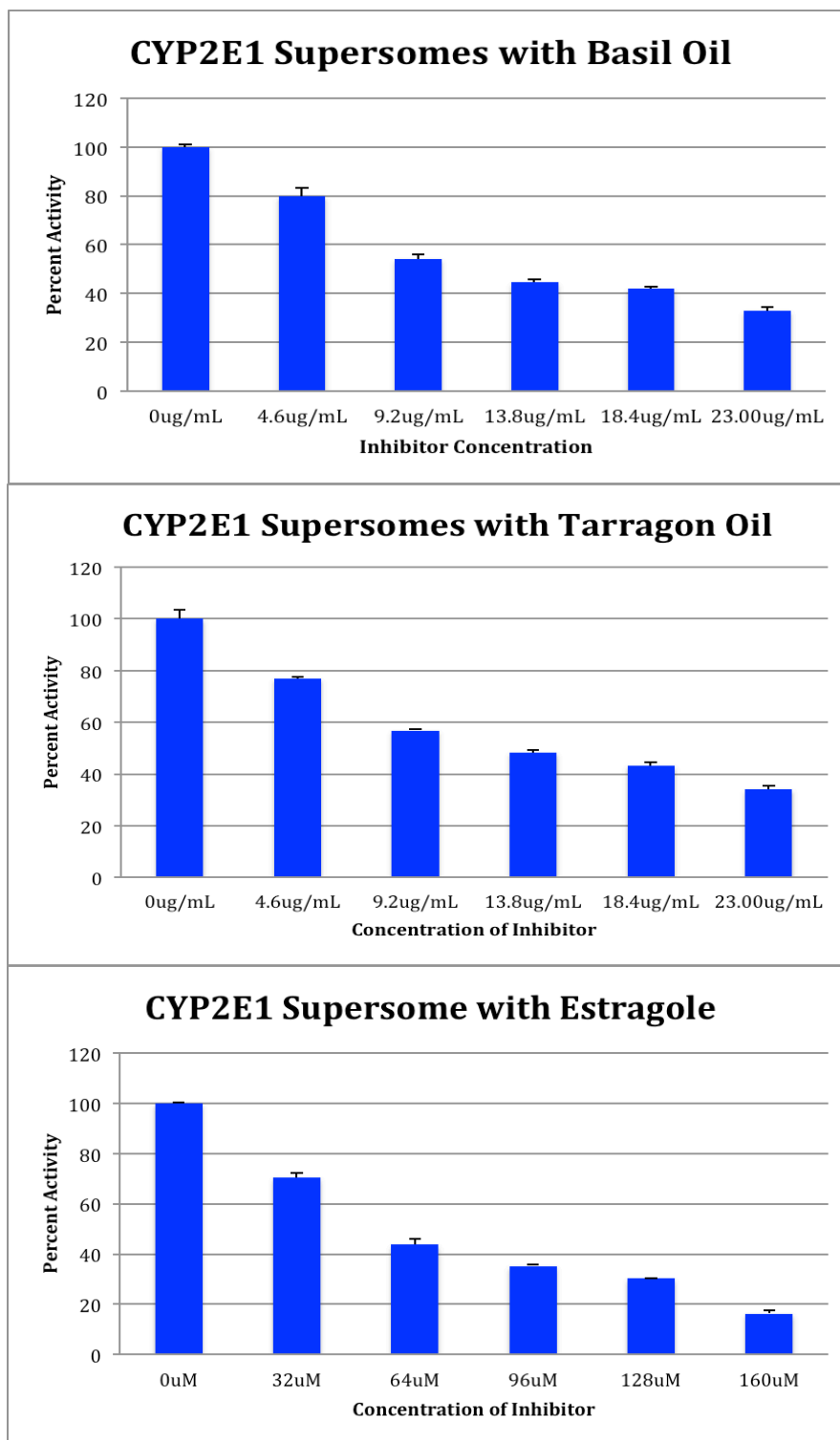


Figure 27-A. Dose Response Studies with Supersomes-CYP2E1.

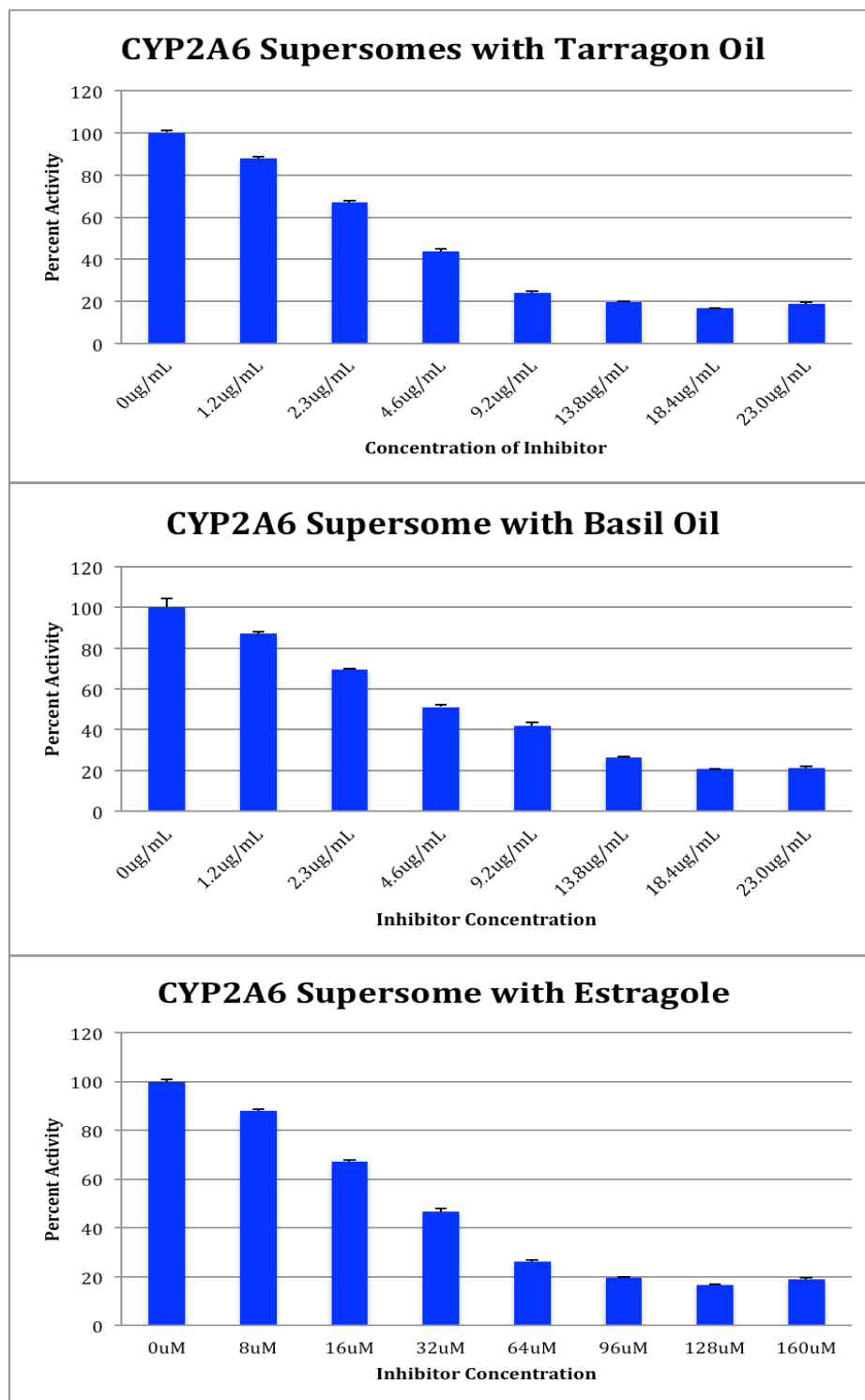


Figure 27-B. Dose Response Studies with Supersomes-CYP2A6.

With CYP2E1 supersomes, 50% of the inhibition with tarragon occurred with 9.2 µg/mL, with basil ~13.2 µg/mL, and with estragole 64 µM. With CYP2A6 supersomes, 50% of the inhibition with tarragon occurred with 4.6 µg/mL, with basil 9.2 µg/mL, and with estragole 32 µM. The time-dependent studies with the supersomes also showed similar results as the studies done with the human, rabbit, and rat. Figure 28A-B shows the results of this experiment. Here again, the enzyme is not being inactivated over time, again showing that the inhibitors undergo a reversible reaction.

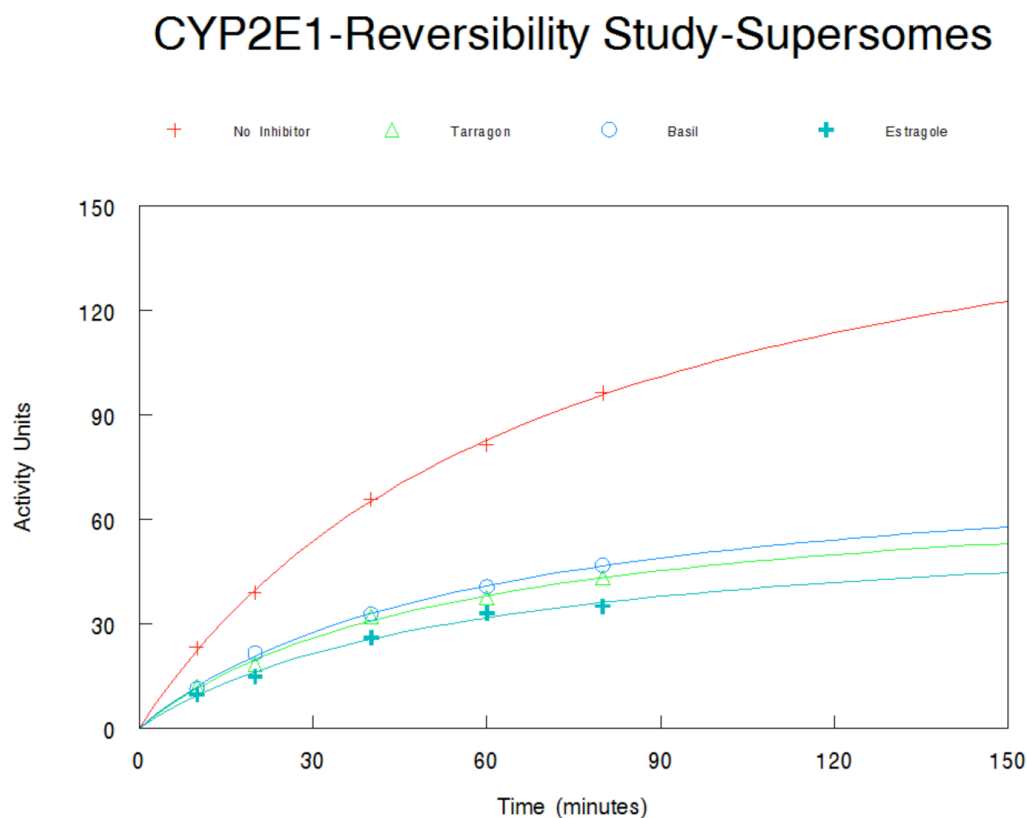


Figure 28-A. Time-Dependent Studies with Supersomes-CYP2E1.

CYP2A6-Reversibility Study-Supersomes

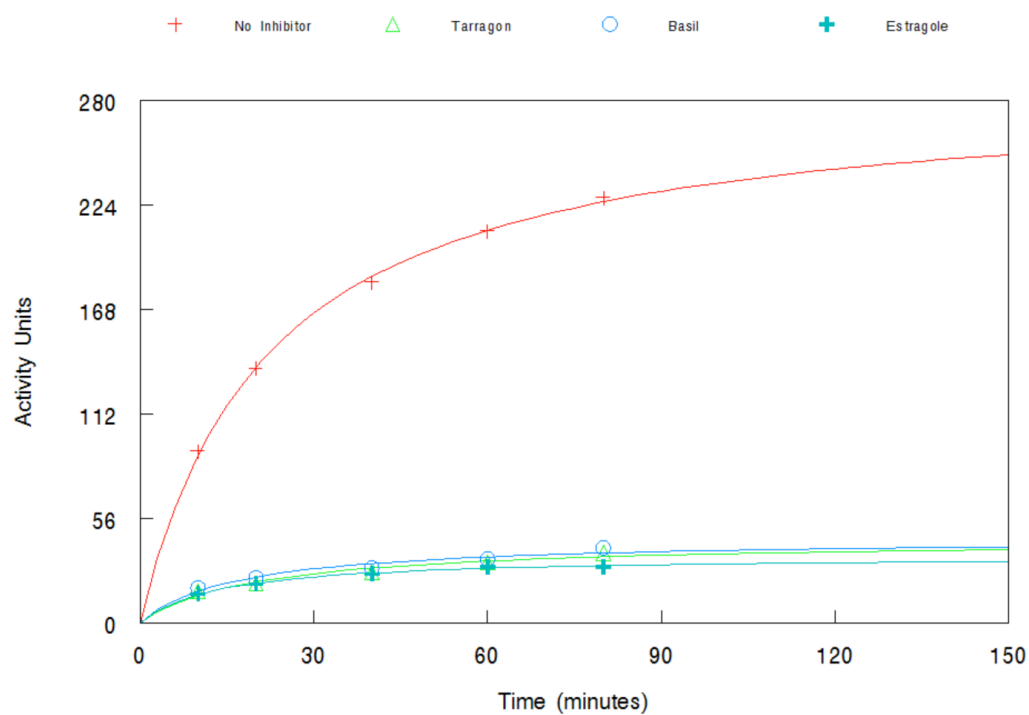


Figure 28-B. Time-Dependent Studies with Supersomes-CYP2A6.

CHAPTER IV

CONCLUSION

This study investigated the inhibitory properties of essential oils on cytochrome P450 enzymes. The findings from this study show that tarragon and basil inhibit both enzymes through a reversible interaction. Estragole being the main constituent within these two oils, it can be said that it is the reason why the inhibition is happening. As predicted the oil with the greater concentration of estragole would inhibit the P450s most. Basil oil has a lower concentration of estragole and this is seen through the results of the studies, thus more of it was required to inhibit enzyme activity by 50%. Tarragon has a higher concentration of estragole, thus required a lower concentration to inhibit the enzyme activity by 50%. Supersomes were also used to confirm the inhibitory affect by these essential oils and the results from those studies followed the same pattern as the liver microsomes of human, rabbit, and rat.

It is important to understand what is happening mechanistically with the reaction. Initially it was thought that the terminal olefin was the reason why the enzyme was being inhibited; as mentioned before terminal olefins are considered “suicide inhibitors” meaning that the inhibitor reaction with the enzyme would be irreversible. As the data has shown, this is not an irreversible reaction but a reversible one. Mechanistically this inhibition reaction could be happening by the estragole coming into the active site of the enzyme (for both CYP2E1 and CYP2A6) with its methoxy pointing inwards and the

terminal olefin facing the opposite direction from the activated oxygen. The methoxy group would then undergo hydroxylation followed by an o-demethylation reaction, producing an alcohol and formaldehyde as an end products. Based on this hypothesis, future studies are aimed at detection of the predicted products of O-demethylation, namely chavicol (*p*-allylphenol) and formaldehyde. This would provide very strong support to the model in which the binding orientation of estragole was with the methoxy group, rather than the olefin, presented to the heme. The active sites of the 2E1 and 2A6 enzymes are also very small and restrictive, unlike most liver P450 isoforms, so rotation of estragole within the confines of this active site may not be possible.

CYP2E1 and CYP2A6 have both been implicated in peroxide production, along with the activation of a number of environmental toxins, which can lead to cancer. It may therefore be beneficial to inhibit these enzymes when certain xenobiotics enter living organisms. The inhibition of these enzymes has been shown with tarragon and basil essential oils, and further studies need to be carried out to determine the mechanism of the reaction to better understand this interaction, along with their potential health benefits. Understanding the metabolism of these essential oils at the mechanistic level will further improve the biochemical knowledge of how these essential oils may help in preventing the deleterious effects of these cytochrome P450 enzymes and the promotion of human health.

According to FEMA the average daily intake of estragole of a 60 kg human is 70 µg, suggesting that the concentration required to inhibit the P450s described in the current study is easily achievable. A study done by Chen and his colleagues in 2007

showed that metabolized products of estragole were detected in humans at concentration of 1 µg/kg²⁷. The physiological relevance of these studies shows that at low concentrations estragole can be potentially bioactivated by P450 enzymes. Furthermore metabolism of estragole by CYP P450 enzymes leads to the formation of 1-sulfooxyestragole, a carbonium ion, which can bind to DNA and proteins and potentially cause cancer.

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